MASTER THESIS

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$P2Y_{11}$ receptors support immune synapse signaling of CD4+ T lymphocytes

By: Sophie Bromberger, BSc
Student Number: 1610692006

Supervisor 1: Dr. Wolfgang G. Junger
Supervisor 2: FH-Prof. MMag. Dr. Veronika Jesenberger

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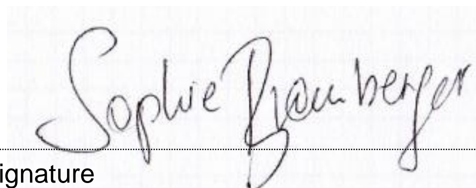

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Kurzfassung

Sepsis ist eine ernstzunehmende klinische Komplikation, die bis heute eine hohe Mortalität aufweist. Sepsis wird durch eine anfängliche hyperinflammatorische Phase charakterisiert, auf die eine T-Zell-basierte Immunsuppression folgt. Dies hat eine höhere Anfälligkeit für sekundäre Infektionen zur Folge. Entzündungsbedingte Gewebeschäden führen zur erhöhten Freisetzung von ATP in den Blutkreislauf, wo es auf Immunzellen wirken kann. Darum ist die Erforschung von purinergen Signalmechanismen ein vielversprechender Ansatz, um der Sepsis-assoziierten Immunsuppression auf den Grund zu gehen. Einige purinerge Komponenten wurden bereits mit der Aktivierung und Funktion von T-Zellen in Verbindung gebracht. Beispielsweise wurde die Regulierung von T-Zellmotilität durch den P2X4-Rezeptor nachgewiesen. Da der $P2Y_{11}$-Rezeptor, neben dem P2X4-Rezeptor, zu den am höchsten exprimierten purinergen Rezeptoren in T-Zellen zählt, zielt diese Arbeit darauf ab, dessen Rolle in der T-Zellantwort genauer zu untersuchen. Jurkat- und primäre CD4+ T-Zellen wurden mit spezifischen Modulatoren des $P2Y_{11}$-Rezeptors sowie seiner Effektoren behandelt. Mit Licht- und Fluoreszensmikroskopie wurden deren Effekte auf T-Zellmotilität, physiologisches Kalziumsignal und die intrazelluläre Lokalisierung von Mitochondrien und P2X4-Rezeptoren analysiert. Zur Quantifizierung der Bildung von Immunsynapsen sowie T-Zellproliferation wurde Durchflusszytometrie mit Antikörperfärbungen verwendet. Sowohl die Migration von T-Zellen, als auch die Co-Lokalisierung von Mitochondrien und P2X4-Rezeptoren wird durch den $P2Y_{11}$-Rezeptor reguliert. Obwohl weder die Bindungskapazität von T-Zellen und Monozyten, noch die basale Mitochondrienaktivität durch den Rezeptor beeinflusst wurden, konnte die mitochondriale Translokation zur Immunsynapse durch die Modulatoren des $P2Y_{11}$-Signalweges bis auf 41% reduziert werden. Auch der charakteristische durch Stimulation induzierte Anstieg im zytosolischen Kalziumsignal konnte inhibiert werden, am stärksten durch den Inhibitor der cAMP-abhängigen PKA. Außerdem wurde auch festgestellt, dass T-Zellproliferation nach Aktivierung durch die Inhibierung oder Überstimulation des $P2Y_{11}$-Rezeptors gehemmt werden kann. Zusammengefasst beweisen diese Daten, dass der $P2Y_{11}$-Rezeptor eine wichtige Rolle in der Signaltransduktion von Immunsynapsen in CD4+ T-Zellen spielt. Dies macht der Rezeptor höchstwahrscheinlich durch die Regulierung der intrazellulären Lokalisierung von Komponenten, die für effiziente T-Zellaktivierung wichtig sind.

Schlagwörter: $P2Y_{11}$-Rezeptor, T-Zellen, Immunsynapse, Mitochondrien, Sepsis

Abstract

Sepsis remains a serious clinical complication with high in-hospital mortality. Characteristically, sepsis is initiated by a mainly neutrophil-mediated hyperinflammatory response, followed by T cell suppression and immunoparalysis, which increases the susceptibility to secondary infections. The associated tissue damage leads to pathologically high ATP concentrations in the extracellular space. Hence, the investigation of purinergic mechanisms in immune cells is a promising approach to identify the cause of sepsis-associated T cell suppression. Several purinergic components have already been identified to regulate T cell function, the latest of which being the P2X4 receptor. Since $P2Y_{11}$ is among the most highly expressed purinergic receptors in human T cells, along with P2X4, this thesis aimed to investigate its role in T cell activation and function. Using specific modulators of $P2Y_{11}$ and its downstream effectors, bright field and fluorescence microscopy were used to measure migration, Ca^{2+} signaling and the localization of mitochondria and P2X4 receptors in Jurkat or primary CD4+ T cells. Flow cytometric methods and antibody staining were performed to observe immune synapse formation and proliferation of T cells upon stimulation. It was shown that inhibition of $P2Y_{11}$ receptors reduces migration, as well as the colocalization of P2X4 receptors and mitochondria in stimulated T cells. Overstimulation of $P2Y_{11}$ receptors surprisingly had similar effects. Despite the fact that neither initial immune synapse formation nor mitochondrial activity were affected by the receptor, mitochondrial translocation to the immune synapse could be reduced by up to 60% compared to the control by modulating the $P2Y_{11}$ pathway and its downstream effectors. Similarly, the physiological Ca^{2+} response to stimulation was hampered upon interference with $P2Y_{11}$, especially when inhibiting the cAMP-dependent PKA. Finally, proliferation following activation was also reduced in CD4+ T cells treated with the respective inhibitors. These data suggest that the $P2Y_{11}$ receptor facilitates signaling at the immune synapse in CD4+ T cells, likely by controlling the intracellular location of components essential for efficient T cell activation.

Keywords: $P2Y_{11}$ receptor, T cells, immune synapse, mitochondrial translocation, sepsis

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1 Introduction

Severe burns and traumatic injuries are the leading cause of death in patients between the ages of 1 and 46, since these conditions usually cause multiple clinical complications [1]. Excessive tissue damage initiates massive inflammation and activation of leukocytes, which can lead to multiple organ failure [2]. At the same time, counter-regulatory anti-inflammatory responses – and in particular the suppression of T cell functions – impair the ability of the host immune system to fight invading microorganisms, thus increasing the risk of sepsis. Sepsis is associated with multiple organ dysfunction syndrome (MODS) that is a major cause of death in trauma patients [1], [3]. According to the World Health Organization (WHO), about 30 million patients worldwide are diagnosed with sepsis each year, with an estimated mortality rate of approximately 15% [4]. Despite intensive research efforts over the last decades, sepsis has remained an unresolved problem and all clinical trials targeting the initial hyperinflammatory response have failed so far [5]. One of the factors complicating the treatment of sepsis is its heterogeneity, with a broad spectrum of symptoms, etiologies and severity [6]. It is therefore of utmost importance to improve the understanding of the exact mechanisms behind the dysregulated immune response in trauma and sepsis patients. Particularly the dysregulation of T cell function in this clinical setting is highly relevant and has been investigated in various *in vitro* and *in vivo* approaches [7]–[14]. One of those approaches has focused on purinergic signaling, which is increasingly being recognized as an important regulator of immune cells [15], [16]. Although previous research in the field of purinergic signaling has focused on neuronal signaling, it has been shown that there are similarities between purinergic signaling of neurons and immune cells, especially since T cells form an immune synapse that resembles the neurological synapse in many aspects [17]. In order to investigate the relevance of purinergic signaling mechanisms in T cell activation, deeper knowledge about these processes needs to be established.

1.1 T cell biology

1.1.1 T cell development

Lymphocytes can be divided into three subpopulations: T cells, B cells and natural killer cells, all of them carrying out distinct functions in the immune response. T cells contribute to the cell-mediated cytotoxic adaptive immune system, with the main function being the elimination of host cells that are either virus-infected or cancerous. The more prominent type of T cells are effector T cells, which either directly exert cytotoxic effects (CD8+), or indirectly facilitate target elimination (CD4+), for example by recruiting other immune cells. Another less abundant T cell subgroup is termed regulatory T cells, which are responsible for controlling auto-reactive T cells [18]. Complex developmental steps and systematic selection mechanisms lead to mature T lymphocytes with a strong affinity for foreign antigens and a weak affinity for self-antigens [19], [20]. T cells share their origin, the

hematopoietic stem cell, with all other cells in the blood. Lymphatic progenitor cells migrate from the bone marrow to the thymus and differentiate into a different population of thymocytes. At this stage they lack both CD4 and CD8 markers and are therefore called double negative (DN). They undergo several DN phases in which they stop expressing CD44 and become CD25 positive. At this stage, two lineages of T cells diverge, according to the organization of their heterodimeric T cell receptor (TCR): the more common α/β T cells and the rarer γ/δ T cells. The heterogeneity of binding affinities of the various T cells is caused by genetic recombination of the TCR subunits [21]. These progenitor cells further proliferate and start expressing CD4 and CD8 co-receptors. At this stage T cells are referred to as double positive (DP) [20]. Whether a cell eventually becomes a single positive (SP) CD4+ or CD8+ T cell, depends on which class of major histocompatibility complex (MHC) they are reactive with: MHC class I recognition contributes to a CD8-committed cell and the recognition of MHC class II initiates CD4 commitment (**Figure 1**) [22].

In the next phase, T cells simultaneously undergo two distinct selection processes termed positive and negative selection. During positive selection, T cells are tested for their ability to interact with the MHC of antigen presenting cells (APC). Additionally, weak binding properties between the TCR and self-antigens are essential for thymocyte survival in this phase [20]. The cortical and medullary thymic epithelial cells (cTECs/mTECs) that act as APCs are strategically positioned to have a higher probability of encountering developing T cells. The specialty of these cells is their capability to express numerous surface molecules of differentiated cells throughout the body. This way the T cells are not only screened for antigens within the thymus itself, but also for a repertoire of other antigens they could potentially encounter in the periphery [23]. In contrast, negative selection is a mechanism of eliminating T cells that have a strong affinity to self-antigens presented by cTECs/mTECs. T lymphocytes that are highly auto-reactive are forced to undergo apoptosis, and dysfunction of this mechanism can increase the risk of auto-immune diseases [23], [24]. Only 1% of T cells, each with an affinity to a specific foreign antigen, survive both of the selection processes and mature further to migrate into the peripheral blood and lymph nodes (**Figure 1**) [25]. This intricate selection mechanism ensures a functional immune system that not only attacks foreign material but also tolerates host tissue.

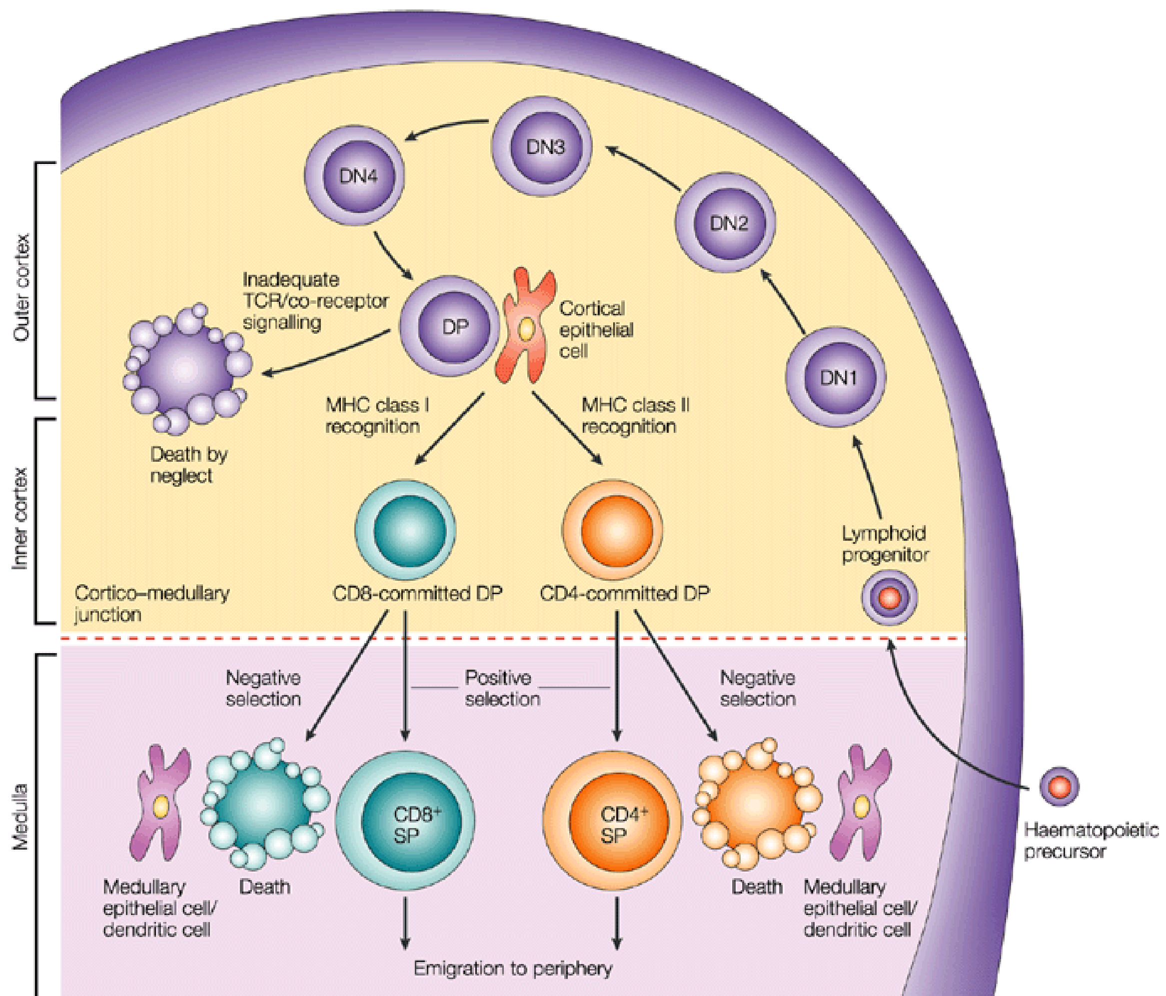


Figure 1: **Development and maturation of T lymphocytes in the thymus**. Lymphoid progenitor cells enter the thymus and undergo several DN phases. Their encounter with cTECs and mTECs triggers co-receptor commitment and determines cell fate via positive and negative selection mechanisms. The mature T lymphocytes, either CD4 or CD8 single positive, then emigrate to the periphery to fulfill their respective functions [22].

1.1.2 T cell activation

Once mature T cells leave the thymus, they patrol the periphery, and remain most commonly in lymph nodes, until they receive a stimulus for activation, specifically the presentation of a foreign peptide by APCs. T cells only recognize the presented antigen if it matches their TCR with high affinity and is presented by APCs. This meticulous selectivity is facilitated by kinetic proofreading, a mechanism that decreases the likeliness of unspecific binding events and activating downstream signaling pathways [26].

Upon ligation of the TCR, the whole contact site undergoes a biochemical transformation that initiates major downstream signaling cascades, as seen in **Figure 2**. First, the Lck tyrosine kinase triggers TCR signaling via phosphorylation of tyrosine residues on the TCR-associated cytoplasmic CD3 and TCR-ζ subunits that share a common immune receptor tyrosine-based activation motif (ITAM) [27]. This event promotes the recruitment of another protein tyrosin kinase (PTK), namely ZAP-70, that is crucial for further intracellular signal transduction [28]. In its activated state ZAP-70 phosphorylates Vav, which subsequently leads to the activation of protein kinase Cθ (PKCθ) and phospholipase Cγ1 (PLCγ1). This induces calcium (Ca^{2+}) release, calmodulin and calcineurin activation and subsequently activation of the mitogen-activated protein kinase (MAPK) pathway that stimulates pro-inflammatory transcription factors like the nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB). In combination with activator protein 1 (AP1) these factors initiate the transcription of pro-inflammatory genes like *IL2*, which codes for interleukin-2 (IL2) [29]. Ultimately, the activated T cells proliferate in order to augment the number of effector cells with an affinity for the respective antigens [30].

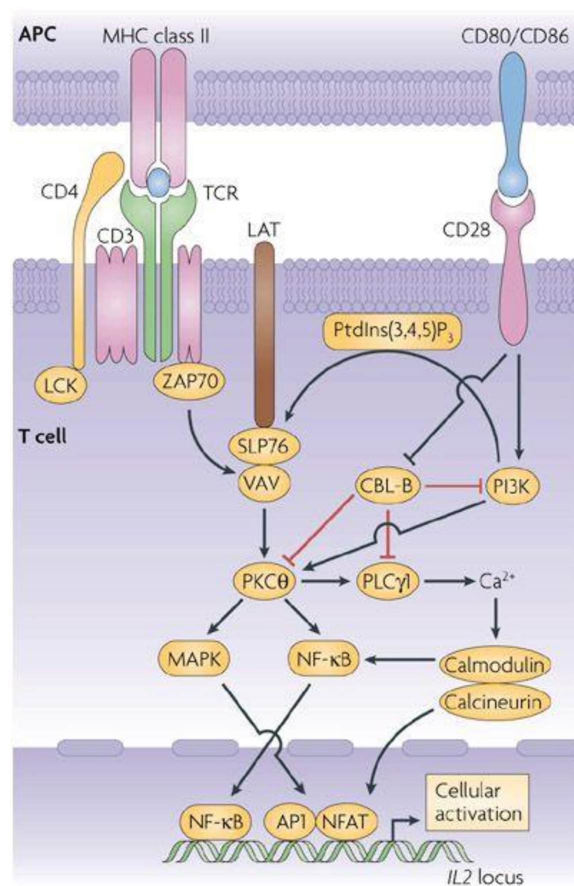


Figure 2: **Downstream targets of T cell receptor signaling.** Upon a TCR ligation event, several downstream kinases including Lck, ZAP-70, Vav, PKCθ and MAPK are consecutively activated, leading to the transcription of *IL-2* [29].

The CD28 co-receptor binds to either CD80 or CD86 of the APC and provides co-stimulatory signals for differentiation and proliferation. Simultaneously, CD28 inhibits the ubiquitin-protein ligase CBL-B (named after Casitas B-lineage Lymphoma), an enzyme that functions as a negative regulator of T cell activation by ubiquitinylating PKCθ and PLCγ1, thus inhibiting the downstream signal transduction and the transcription of activation-associated genes (**Figure 2**) [31].

Studies have shown that cyclic AMP (cAMP) is an important second messenger with potent negative regulatory effects of TCR-mediated T cell activation [32]–[34]. Increase in cAMP activates protein kinase A (PKA), which in turn activates Csk and inhibits Lck and other key signaling elements downstream of TCR/CD28. It is believed that regulatory T cells transfer cAMP via gap junctions to effector T cells in order to restrict their activation [35]. This mechanism is crucial for T cell regulation to avoid inadequate activation, but when imbalanced, the cAMP/PKA inhibitory signaling pathway can result in dysregulation of T cells and therefore have detrimental functional consequences [34].

1.1.3 The immune synapse

When a T cell encounters an APC carrying the matching antigen, both cells form a highly specialized structure, termed the immune synapse (IS). The term is derived from neuronal synapses, as both share common characteristics including molecular recognition events, positional stability and directed, secretory communications [17], [36], [37]. Upon recognition of matching antigen, the T cell forms a transient, dynamic structure of receptors and co-receptors around the TCR-MHC contact, as shown in **Figure 3**. This structure promotes optimal antigen recognition and further downstream signaling. Co-receptors of the TCR include CD3 and CD28 as well as CD4/CD8, CD43-45, CD2 and several other molecules (**Figure 3a**). A TCR-dependent transactivation of adhesion molecules like integrins is necessary to maintain the stability of the IS [38]. The three-dimensional (3D) structure of the IS assures close proximity of the associated receptors and co-receptors, so that only a small part of the cell surface has to flatten and engage in the process. This bull's eye arrangement of so-called supramolecular activation clusters (SMACs) forms within a few minutes of T cell-APC contacts [39]. As can be seen in **Figure 3b**, there is a central SMAC (cSMAC) that is enriched with TCR MHC-peptide complexes and its downstream targets. The circular proximal SMAC (pSMAC) contains co-receptors like lymphocyte function-associated antigen 1 (LFA1), which binds to intercellular adhesion molecule 1 (ICAM-1) on the APC [40]. Additionally there is the dorsal SMAC (dSMAC), consisting primarily of CD43-45 molecules, which separate the inside of the synapse from the extracellular environment while also ensuring structural stability (**Figure 3b**) [41], [42].

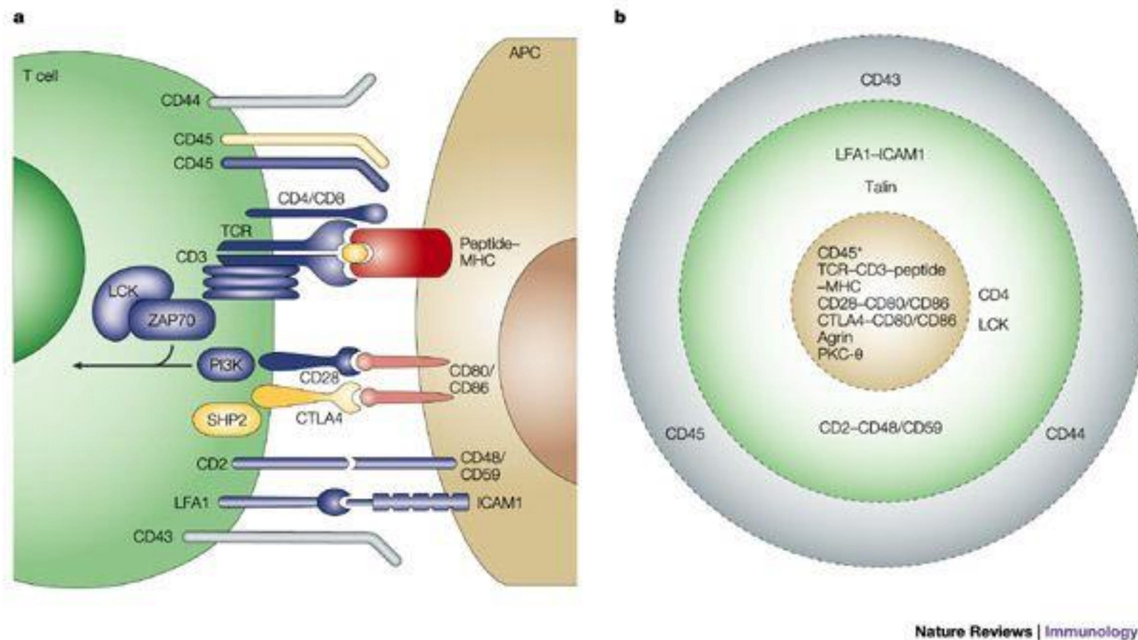


Figure 3: **Structure of the immune synapse**. When a T cell recognizes cognate antigen on an APC, it forms a transient, dynamic structure. (a) Both cells flatten at the recognition site to increase the area of surface contact. Besides the TCR-MHC-peptide complex, numerous other T cell receptors and ligands play a role in forming the IS, including CD28 with its respective binding partner CD80/CD86, CD43-45 and CD2 binding to CD48. (b) The bull's eye arrangement, including the central, proximal and dorsal SMAC, allows for close proximity of associated receptors and ligands, which ensures efficient signal transduction [43].

During formation of the IS, the cytoskeleton as well as several intracellular organelles, including mitochondria and the endoplasmic reticulum, reorganize within the T cell to facilitate the signaling pathways leading to activation [44], [45]. This process is also referred to as polarization. When polarizing, T cells reorganize their components to form the anterior region termed the leading edge, and the posterior trailing edge or uropod. While all elements necessary for sufficient activation translocate to the leading edge, the majority of other organelles and molecules accumulates at the uropod [46]. Actin filaments and microtubules are affected in particular, as they are coordinately rearranged. During activation, actin filaments tend to accumulate near the synapse, to be able to constrict the lamellopodia that form around the APC. This ensures proper maturation and stability of the IS [47]. The centrosome, also called the microtubule organizing center (MTOC) in this context, translocates from the perinuclear space toward the cSMAC, where it orchestrates the secretion of microvesicles containing cytokines [48]. The MTOC furthermore facilitates the transport of essential components for IS formation along the microtubules [49]–[51]. For example, the mitochondria are coupled to the microtubules via the so-called motor adaptor. This involves several proteins anchored to the outer mitochondrial membrane (OMM), and adaptor proteins that build the link between the motor proteins and the anchorage [52]. One

of these adaptor systems involves the OMM-bound mitochondrial Rho-GTPase (Miro1/2) and an adaptor protein called trafficking protein kinesin-binding (Trak1/2), which can bind to both kinesin or dynein [50], [52]. Miro1 possesses two EF hand domains on its cytoplasmic domain, which upon binding of Ca^{2+} induce a conformational change releasing Miro1 from its adaptor protein. Without Ca^{2+} bound, Miro1 connects the mitochondria to the cargo-binding terminus of kinesin or dynein via Trak1 or Trak2, respectively [53]. This mechanism is also regulated via the phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1), which ubiquitinylates Miro1 and therefore marks it for degradation. Thus, cells tightly control the trafficking of these essential organelles to the immune synapse in order to provide adenosine triphosphate (ATP) and facilitate Ca^{2+} signaling [54], [55]. In this setting, the spatio-temporal control of Ca^{2+} signaling in different microdomains is especially important [56]. Additionally, mitochondria are key players in proliferation by contributing to reactive oxygen species (ROS) signaling and regulating effector/regulatory T cell development through glycolysis or ß-oxidation, respectively [57].

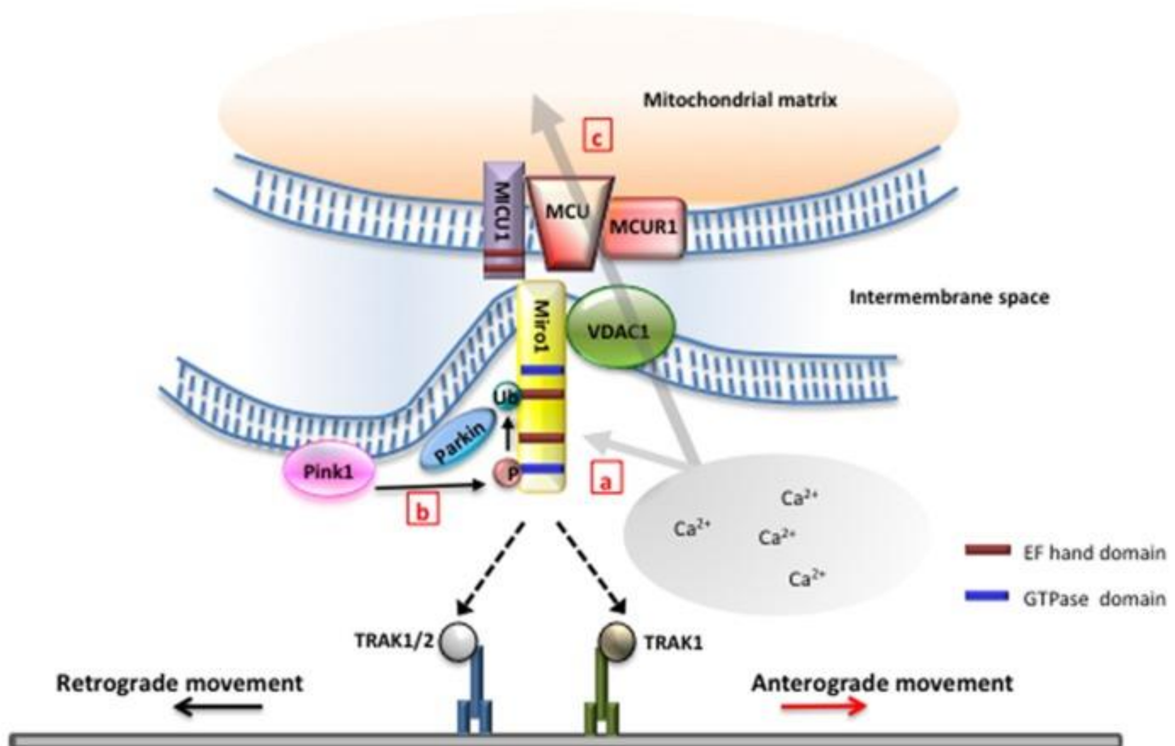


Figure 4: The mitochondrial motor adaptor complex. Miro1 binds to Trak1/2 and links the mitochondria to motor proteins (kinesin or dynein). Upon binding of Ca^{2+} to the EF hands of Miro1, a conformational change is induced and releases the protein from its adaptor. Another mechanism of regulating Miro1 is by tagging it for degradation via ubiquitinylation, which is performed by PINK1 and Parkin [53].

1.1.4 T cell migration and function

T cells that have not yet encountered antigen are referred to as “naïve” T cells. Once a naïve T cell binds to its particular antigen, it will start proliferating to create a larger population of cells with the same specificity. In this phase, T cells also augment their number of mitochondria in order to meet the high demands of energy during proliferation [58]. These cells then acquire effector functions and migrate toward the site of inflammation to seek the cognate antigen [59]. Optimal immune surveillance is ensured by lymphocyte trafficking within the blood, as well as lymphoid and non-lymphoid tissues. In contrast to other leukocytes that migrate in response to unspecific inflammatory signals, T cells have well defined and coordinated trafficking patterns according to their state of activation, differentiation and function. Naïve T cells generally recirculate within the secondary lymphoid tissues to search for matching antigen, while primed T cells achieve the ability to specifically identify and migrate towards sites of antigen accumulation in non-lymphoid tissues [60]. Extravasation of lymphocytes is highly dependent on distinct sets of chemokines and adhesion molecules expressed on the surface of the vascular endothelial cells within a certain tissue or organ. For example, homing of primed T cells to sites of inflammation in the skin have been linked to the expression of the CC-chemokine receptor 4 (CCR4), CCR8 and CCR10 [61]. Meanwhile, the stromal derived factor 1α (SDF-1α, also known as CXCL12), which binds to the CXCR4 receptor on CD4+ T cells, contributes to homing and migration within lymph nodes [62]. Because of the strong dynamic forces in blood vessels, particularly the shear stress, immune cells need mechanical anchors to firmly attach to the vessel walls and be able to extravasate. These mechanical anchors belong to the family of selectins, which are present on leukocytes (L-selectins), thrombocytes (P-selectins) and endothelial cells of the vessel wall (P- and E-selectins) [63]. The binding of selectins is, however, not sufficiently resistant to the shear stress of the blood flow, which is why the leukocytes roll slowly along the endothelial wall. A complete halt of cell rolling can only be achieved when a second anchoring system based on integrin molecules is triggered [59]. Prominent examples of additional key molecules are LFA-1 which binds to the endothelial ICAM-1, as well as Vav proteins which are also involved in T cell activation, as described earlier [64].

Once arrived at the target, the effector T cells have to carry out their respective functions. Upon encounter of their corresponding antigen CD8+ cytotoxic T cells secrete a range of cytotoxins like perforin, granzymes and granulysin. Perforin binds to the target cell membrane and forms pores to enable the entry of pro-apoptotic serine proteases called granzymes into the target cell. Those subsequently trigger the caspase cascade, leading ultimately to apoptosis of infected or cancerous host cells [65]–[67]. Meanwhile, CD4+ T cells, also referred to as T-helper cells, assist the cytotoxic T cells by secreting cytokines like IL-2, interferon-γ (IFN-γ) or tumor necrosis factor β (TNF-β). These cytokines further mediate the activation of cytotoxic T cells, but also stimulate macrophages and other

immune cells [68]. The majority of effector cell clones dies after clearance of the respective antigen, but a small fraction survives as memory T cells and returns to the periphery for host protection. Should the same antigen infiltrate the host again, an even faster immune response can be orchestrated with the help of these memory T cells [69].

1.2 Purinergic signaling

The purine nucleotide ATP is the universal unit of cellular energy transfer. It is involved in most cellular processes requiring energy stored in its phosphoanhydride bonds. ATP is produced either via glycolysis or in mitochondria, through oxidative phosphorylation and the electron transfer chain that uses the chemiosmotic potential of an H+ proton gradient to generate ATP from adenosine diphosphate (ADP). While these intracellular mechanisms are well-characterized, the importance of ATP and its metabolites as extracellular signaling molecules has only recently been discovered. When ATP is released into the extracellular space, for example via membrane channels or vesicular transport, it can bind to purinergic receptors expressed on the surface of virtually all mammalian cells, triggering diverse downstream signaling cascades. Even though the main focus of purinergic signaling lies on ATP and its metabolites, they are not the only molecules to be considered. Uracil triphosphate (UTP) and its metabolites are structurally similar to ATP and several purinergic receptors respond to them as well [70]. While ATP was thought to be leaking into the extracellular space only upon cell death, it has been first shown in neurons that viable and intact cells can also intentionally release ATP [71]. Since then, studies demonstrated the importance of extracellular purinergic signaling in various other tissues like the vasculature, liver, muscle, eyes, skin, gastrointestinal tract and the immune system [72]–[78].

1.2.1 ATP release and extracellular metabolism

Known membrane channels for ATP release are members of the pannexin (Panx) hemichannel family [79]. There are three subtypes of pannexins (Panx1-3), with Panx1 being by far the most widespread among tissues and therefore also the most well-studied of the three. Panx proteins presumably form hexameric (Panx1 and 3) or octameric (Panx2) ion- and metabolite-permeable channels that can regulate ATP efflux. They often seem to be coupled to different ligand-gated membrane receptors, which favors distinct autocrine/paracrine signaling mechanisms [80], [81]. These receptors are mostly located at the plasma membrane of a cell, where they mediate intercellular communication circuits [82]. However, it is believed that functions of Panx are not limited to the cell surface, but that these channels can in fact also be located at the membranes of intracellular compartments. It has been shown that high concentrations of extracellular ATP can directly or indirectly induce the internalization of Panx1 receptors [83], [84]. The precise intracellular functions of Panx1 remain yet to be elucidated. Panx channels can be activated by diverse signals. In neutrophils, for example, the stimulation of the formyl peptide receptor (FPR) triggers

Panx1-mediated ATP release [85]. Other mechanisms of Panx activation include mechanotransduction and elevated levels of certain ions like intracellular Ca^{2+} or extracellular potassium (K^{+}), as well as phosphorylation by Src kinases [86].

Not only is ATP intentionally released into the extracellular space, but also coordinately metabolized outside the cell (**Figure 5**). Ectonucleotidases bound to the cell membrane can hydrolyze extracellular ATP to form ADP, AMP and adenosine. Ectoenzymes involved in this process are ectonucleoside triphosphate diphosphohydrolases (ENTPDs, e.g., ENTPD1 or CD39), ectonucleotide pyrophosphatase/phosphodiesterases (ENPPs), alkaline phosphatase (AP) and ecto-5'-nucleotidase which is also referred to as CD73 [87]. These ectoenzymes can be found on most mammalian cells, organized in tissue-specific distribution patterns to support optimal signaling. Extracellular adenosine can either be converted into inosine by adenosine deaminase, or re-internalized by concentrative nucleoside transporters (CNTs) or equilibrative nucleoside transporters (ENTs). Intracellular adenosine can then be reused for ATP production [88], [89].

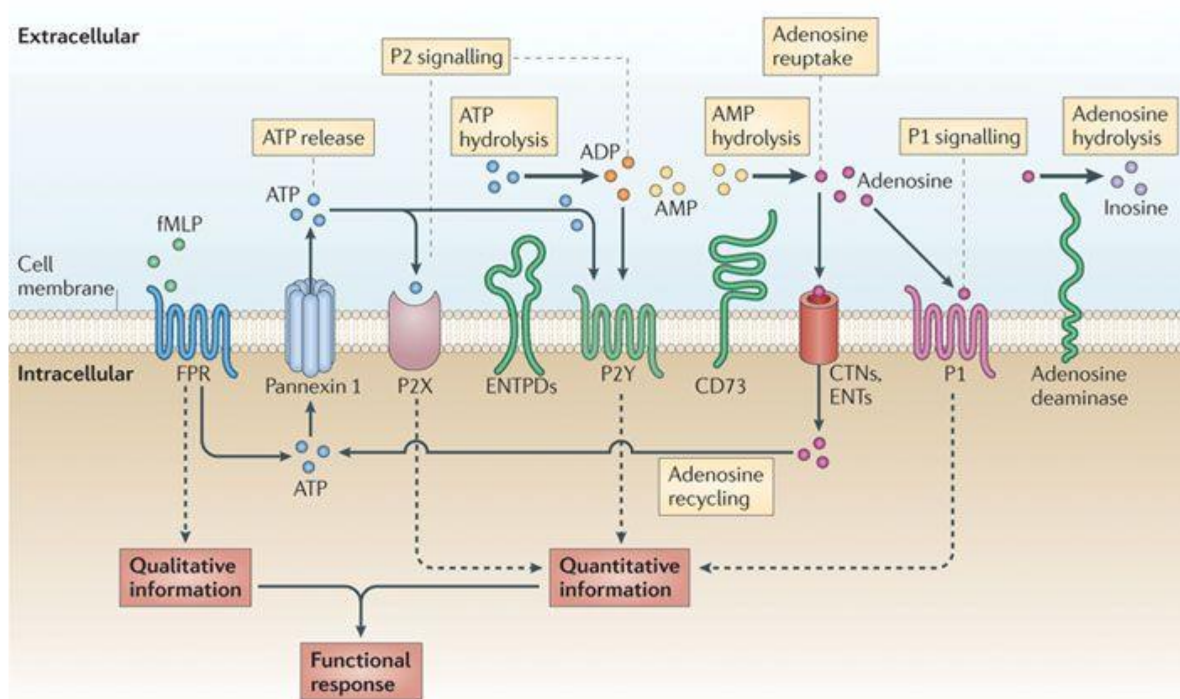


Figure 5: Key components of autocrine purinergic signaling. ATP is released via Panx1 hemichannels after the stimulation of a cell surface receptor (e.g. FPR in neutrophils) and either stimulates P2 receptors directly, or undergoes hydrolysis by ENTPDs to be degraded into ADP or AMP, respectively. While ADP can activate P2Y receptors, AMP is degraded by CD73 into adenosine, which stimulates P1 receptors. Reuptake of adenosine is accomplished by CNTs or ENTs and intracellular adenosine can then be used for further ATP production. Adenosine deaminase can metabolize extracellular adenosine to inosine [78].

1.2.2 Purinergic receptors

Extracellular ATP and its metabolites can bind to specialized membrane receptors in order to induce an intracellular signaling event. The effect of activating a purinergic receptor is not only dependent on the nature of the receptor itself but also highly influenced by the cell type carrying the receptor. Since purinergic signaling involves ubiquitous molecules like ATP, Ca^{2+} and G proteins, all of them being mediators in countless cellular processes, the downstream targets are quite diverse. There are two main groups of receptors, namely the P1 and P2 receptors. P1 receptors are G-protein coupled receptors (GPCRs) that recognize adenosine and can be further distinguished into A_1, A_{2A}, A_{2B}, and A_3 subtypes, each one fulfilling distinct functions [90]. For example, the ligation of A_1 or A_3 receptors inhibits adenylyl cyclase activity and therefore the production of cAMP. Meanwhile, A_{2A} and A_{2B} receptors can, upon activation, upregulate intracellular cAMP levels via stimulation of the cAMP–dependent PKA [91]. The importance of cAMP as a pivotal second messenger in countless cellular processes implies the relevance of adenosine and P1 receptor signaling.

The P2 receptor family can be further divided into P2X and P2Y receptors, which respond to ATP or other nucleotides. P2X receptors are ATP-gated ion channels that promote influx of extracellular cations, particularly Ca^{2+}. They can be classified into 7 subtypes (P2X1-7) which have a widespread tissue distribution. Although there has already been a lot of research on these receptors in neurons, they are currently being studied in many other tissues and cell types including liver, bone and muscle as well as several forms of cancer [92]–[97]. For example, P2X7 is a receptor of interest due to its unique ability to open a non-selective plasma membrane macropore upon overstimulation with ATP [98]. This is a particularly attractive topic in cancer and inflammation research. In contrast to the P2X receptors, the P2Y family does not bind exclusively to ATP but also to ADP, UTP and UDP. To date, eight subtypes of P2Y receptors ($P2Y_{1, 2, 4, 6, 11\text{-}14}$) have been identified and their structure resembles P1 receptors since they also function as GPCRs [99]. A prominent example for a clinically targeted P2Y receptor is $P2Y_{12}$. This receptor is coupled to the G_i protein in platelets and suppresses adenylyl cyclase. Therefore the fibrinogen-receptor glycoprotein IIb/IIIa (GPIIb/IIIa) is not inhibited, which is substantial for platelet assembly [100], [101]. Previous studies have shown that mutations in the *P2RY12* gene are causing congenital bleeding disorders due to the disruption of correct platelet aggregation [102], [103]. In contrast, increased risk of ADP-induced platelet aggregation was observed in subjects with gain-of-function mutations of *P2RY12* [104]. Due to these properties, the $P2Y_{12}$ is already used clinically as a target for clopidogrel, a common anticoagulant used to treat and prevent vascular ischemic events in patients with atherosclerosis or acute coronary syndrome [105].

1.2.3 Purinergic signaling in the nervous system

As mentioned earlier, the mechanisms of purinergic signaling were most thoroughly studied in neurons, where they were first observed in the 1970s [106]. Since then, it has been shown that ATP is a co-transmitter in all nerve types. It is not only a part of the conduction of action potentials along peripheral neurons, but also acts in supportive cells like astrocytes and glial cells. Not surprisingly, purinergic signaling has been proven relevant in several neurodegenerative diseases like Alzheimer's, Parkinson's, or amyotrophic lateral sclerosis [107]–[110].

Figure 6 outlines the distinct functions that ATP and its receptors have to fulfill in the central nervous system. Alongside well-known neurotransmitters like Ca^{2+} that are active in the synaptic cleft, ATP and ADP are secreted into the synapse via vesicular transport. Activating P2X2, P2X4 and P2X6 receptors on the postsynaptic neuron, this process supports the transduction of action potentials from one cell to the other (**Figure 6**) [71]. Neuronal-glial communications are partly mediated by ATP as well, since it activates respective intracellular Ca^{2+} signaling via P2 receptors (e.g. P2X7, $P2Y_{12}$) on macro- and microglia. The activation of microglia can then initiate neuron-protective events via ATP-mediated stimulation of astrocytic $P2Y_1$ and $P2Y_2$ receptors [111], [112]. The $P2Y_1$ receptor also triggers the myelination process in oligodendrocytes, constituting another neuroprotective mechanism [113]. Meanwhile, recent studies investigate $P2Y_2$ for its role in nerve and spinal cord regeneration [114], [115].

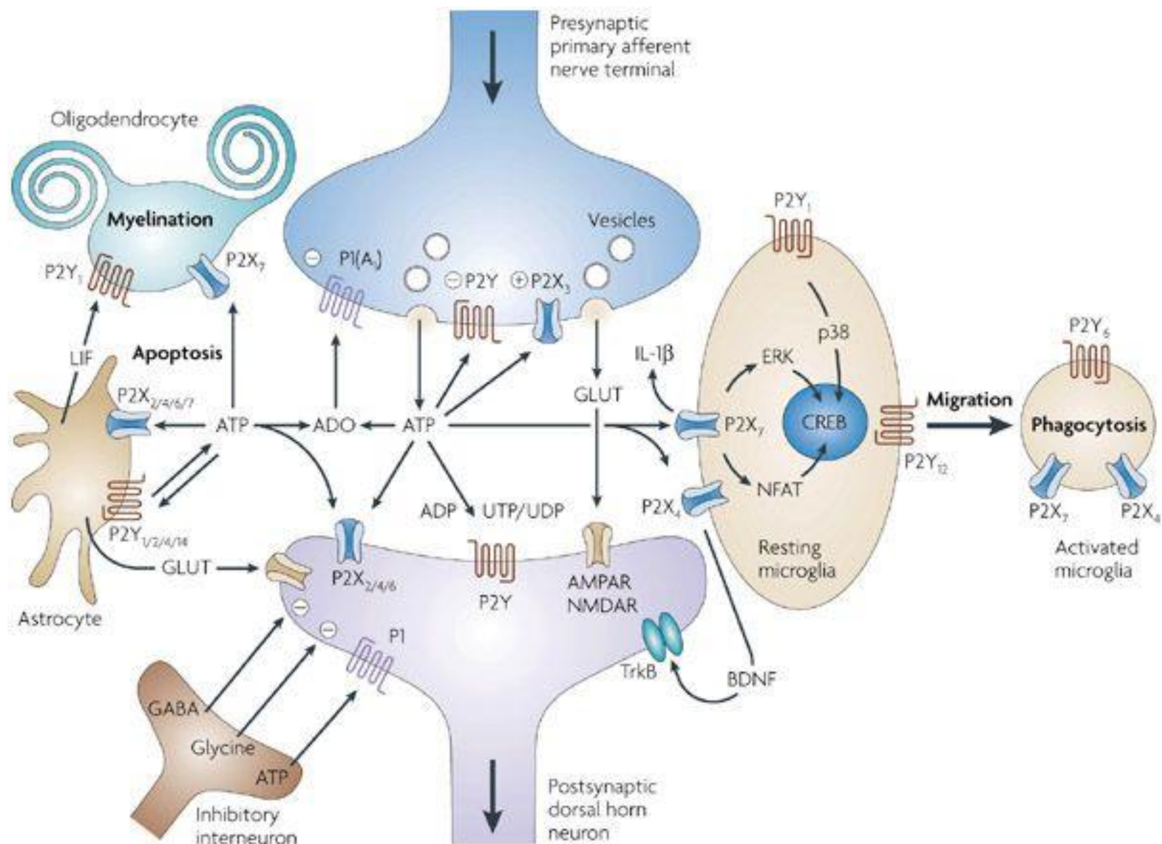


Figure 6: Purinergic signaling in the central nervous system. This figure shows the distinct roles of ATP and its receptors in different parts of the nervous conductive system. The neurological synapse in the center relies on numerous receptors for signal transduction, as do other cells involved like astrocytes or microglia. Neuronal-glial communications as well as other neuroprotective mechanisms have been shown to be mediated by components of the purinergic system [71].

1.2.4 Purinergic signaling in the immune system

Over the past two decades an increased interest in the role of purinergic signaling in the immune system has formed. Current literature indicates that nucleotide-dependent signaling mechanisms might play a vital part in immune regulation. ATP is released in high doses upon cell or tissue damage and acts as a damage-associated molecular pattern (DAMP) to inform the immune system that damage has occurred [116]. Additionally, ATP and adenosine have been shown to contribute to chemotaxis, therefore also guiding the immune cells toward the location of that event [117], [118]. Studies suggest that ATP and UTP are important factors in the migration of hematopoietic stem cells as well [119]. From an immunological perspective, ATP signaling (via P2 receptors) is generally regarded as pro-inflammatory, while adenosine signaling (via P1 receptors) works mainly in an anti-

inflammatory manner [120], [121]. However, ATP can also hamper the activation of immune cells, specifically when released in lower doses over a long period of time [122], [123].

Regarding the clinical applications of this knowledge, the components of purinergic signaling are already being investigated as potential therapeutic targets. An especially advantageous property of purinergic receptors is their accessibility at the cell surface, which enables easy modulation. Since this system has such widespread functions throughout many tissues and cell types, a lot of information remains yet to be elucidated. This is also the reason why there are only a few clinically approved drugs involving the purinergic pathways yet. Many approaches try to utilize the anti-inflammatory properties of extracellular adenosine or the P1 receptors, respectively. It is believed that patients with chronic inflammatory diseases could benefit from adenosine-based therapeutics [78]. Another substantial goal for the use of purinergic components is in organ transplantation, which generally comes along with immense tissue damage due to the activation of immune cells targeting the donor organ. It has been suggested that factors of the purinergic signaling system contribute to this mechanism. An intervention of this process – to avoid the unwanted recruitment of immune cells to the donor organ – could potentially reduce organ rejection in the future [124]. Furthermore, although it is a relatively young field, efforts have been made to investigate the effects of purinergic signaling in stem cell differentiation and tissue regeneration as well. In stem cell transplants, proliferation and migration of stem cells to the site of injury are crucial for the success of the therapy and the patient's outcome. Researchers suppose that purinergic receptors like $P2Y_2$ could be potential targets for improved regeneration [125].

1.2.4.1 Neutrophils and macrophages

For a proper function of immune surveillance and host defense, neutrophil granulocytes must not only be able to detect minimal amounts of chemoattractants, but also to migrate toward their source, the site of inflammation [126]. This intricate process requires highly organized signal amplification and feedback loops. However, the classical model of inflammatory cell trafficking mediated only by chemokines had to be reconsidered. In 2006, Chen *et. al.* have first shown that ATP is released by neutrophils upon the stimulation of chemotaxis receptors like the FPR1 by N-formylmethionine-leucyl-phenylalanine (fMLP) [118]. Since then, this topic has been investigated more thoroughly. Due to the fact that both purinergic and chemotactic pathways rely heavily on Ca^{2+} as a second messenger, it seems only logical that these processes are intertwined [127]. Purinergic signaling components have been linked to chemotactic pathways by the ability to modulate chemokine secretion [128]. Not only do neutrophils release ATP, they also possess the respective receptors to respond to it and are able to generate extracellular adenosine via CD73 [129]. A more recent study has shown that the F0F1 ATP synthase (FATPase) is a key mediator of the formation of cytoplasmic protrusions and the directed ATP release during neutrophil chemotaxis [130]. The FATPase is capable of translocating to the leading edge of migrating neutrophils, and

colocalizing with Panx1, which adds to the migratory feedback machinery. Additionally, FPRs have been demonstrated to colocalize with $P2Y_2$ receptors on the surface of neutrophils upon stimulation, indicating that purinergic signaling components play an intricate role in neutrophil activation and migration [85]. Other studies have described P1 and P2 receptors to be important migratory factors in diverse clinical settings, including pulmonary fibrosis, gout, breast cancer and sepsis [131]–[134].

The function of monocytes or macrophages, as they are called when they exit the blood circulation and enter other tissues, is also highly dependent on the ability to migrate. Cells that are damaged, necrotic or apoptotic release ATP as a find-me signal for all kinds of phagocytes, including macrophages, to promote phagocytic clearance of cellular debris [135]. Interestingly, it has been shown that monocytes release ATP as well when treated with pathogen-associated ligands, which in turn leads to the secretion of inflammatory cytokines such as IL-1β or IL-18 [136]. This autocrine feedback loop involves $P2Y_2$, $P2Y_{12}$ and P1 receptors and it is essential for chemotaxis [137]. In contrast, it has also been suggested that purinergic metabolites such as adenosine can terminate the macrophage response, thereby avoiding excessive activation of inflammatory cells [138]. This indicates a well-controlled and self-restrictive feedback mechanism which is crucial for proper macrophage migration and function. Targeting these mechanisms therapeutically might be beneficial to patients with several conditions, as it has already been linked with improved survival in murine sepsis models [139].

1.2.4.2 T cells

As mentioned earlier, the key components of T cell activation are the TCR and the MHC carrying a foreign peptide. However, the likelihood of a T cell encountering one or even more APCs carrying their respective antigen is very low and it is surprising how such brief encounters with trace amounts of antigens are strong enough to sufficiently activate T cells. Therefore, the cells need an elaborate system that allows them to act upon minimal concentrations of stimulants. Numerous models have been established to explain how T cells intensify these weak signals in a sufficient way to generate strong activating cues [140]. The idea of autocrine signal amplification circuits has gained more attention after it was proven that T cells release ATP via Panx1 channels upon stimulation [141], [142]. In T cells, P2X1 and P2X4 receptors have been found to translocate along with Panx1 to the immune synapse [143], [144]. Additionally, P2X4 has very recently been identified as an essential regulator of T cell migration [145]. P2X7 is believed to play a role as well, although it does not translocate upon stimulation [143]. These ion channels can increase the influx of Ca^{2+}, which fuels the ATP synthase in the mitochondria to generate more ATP that can be released [54], [145]. Simultaneously, Ca^{2+} activates other downstream processes ultimately leading to the transcription of pro-inflammatory genes like *IL-2*. A graphic demonstration of a signal amplification loop like this can be seen in **Figure 7**, where a stimulus induces the

release of Ca^{2+} from intracellular stores, leading to ATP production and further stimulation of P2X receptors, which in turn allows more Ca^{2+} influx. This creates a signaling circuit that induces a substantial response to a faint initial stimulus [54]. Naïve T cells tend to accumulate in lymph nodes, where this form of signaling also works in a paracrine manner, meaning that the ATP released from one activated T cell can stimulate other cells in close proximity. It has been shown that this process reduces the T cell motility, which in turn allows for more efficient tissue scanning [146].

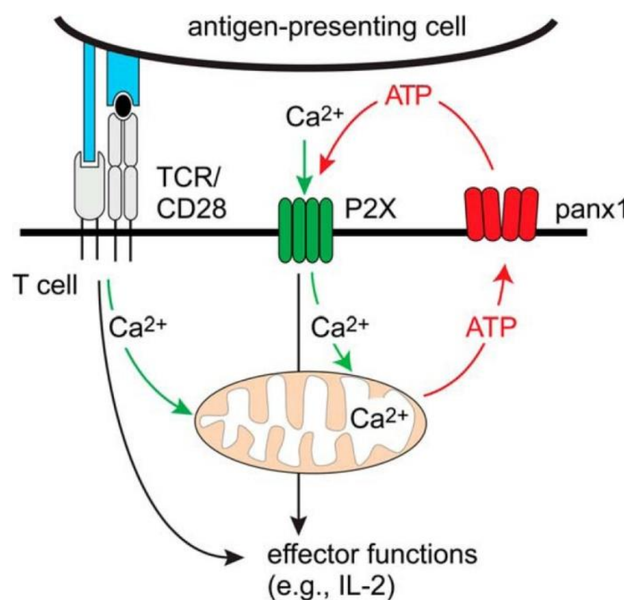


Figure 7: **Autocrine purinergic signaling to amplify T cell activating signals**. TCR ligation triggers Ca^{2+} signaling, which is essential for the ATP synthesis in the mitochondria. ATP is then released via Panx1 and activates P2X ion channels, which leads to more Ca^{2+} influx, stimulating the mitochondria to generate ATP [54].

Interestingly, purinergic components not only modulate the activation of T cells, but also the decision whether to become an effector or regulatory T cell. It has been shown previously, that a high concentration of extracellular ATP and the activation of purinergic receptors inhibits the generation and function of regulatory T cells, while simultaneously promoting the activation of effector T cells [147], [148]. For example, ATP release and P2X7 signaling are important co-stimuli in γδ T cell lineage progression and activation [149], [150]. Similarly, the long-term fitness of CD8+ memory T cells is directed by the P2X7 receptor [151]. However, as mentioned above, purinergic signaling also includes adenosine, which has been shown to have anti-inflammatory effects, in contrast to the pro-inflammatory properties of ATP and ADP. In T cells, adenosine can act as a potent immunosuppressant, especially upon binding to A_{2A} receptors. CD39 and CD73 are highly abundant on the surface of

regulatory T cells, allowing quick degradation of ATP and higher concentrations of extracellular adenosine, which in turn causes the suppression of effector T cells [152]. These findings suggest a sophisticated and self-restrictive feedback mechanism to avoid inappropriate activation of cells.

It is common knowledge that the human immunodeficiency virus (HIV) infects T cells and disrupts the immune competence, which makes the carrier prone to infections. In 2011, HIV infection was linked to purinergic signaling when Séror *et. al.* demonstrated that purinergic receptors facilitate viral entry [153]. Several components of the purinergic system were subsequently shown to have very distinct effects on the viral infection, some even strengthening the defense against HIV [154]. The research on this topic has been very promising so far, resulting in several potential new therapeutics against HIV infection and the acquired immunodeficiency syndrome (AIDS) [155]–[158].

1.2.5 The $P2Y_{11}$ receptor

The $P2Y_{11}$ receptor belongs to the GPCR family and is unique in that it couples to both $G\alpha_{q/11}$ and $G\alpha_s$, which have distinct downstream functions. The activation of $G\alpha_{q/11}$ stimulates PLCβ, which cleaves phosphatidylinositol 4,5-bisphosphate into inositol triphosphate IP3, leading to the release of Ca^{2+} from the endoplasmic reticulum, and diacylglycerol, which activates PKC, respectively. In contrast, the activation of $G\alpha_s$ promotes the activation of adenylyl cyclase, leading to the production of cAMP and subsequently the stimulation of PKA [159]. PKA has several functions in various eukaryotic cell types, but in T cells specifically the cAMP-PKA pathway leads to activation of Csk which in turn inhibits Lck, therefore ultimately inhibiting further downstream mechanisms of T cell activation [34]. Since the downstream targets of $P2Y_{11}$ involve two universal second messengers, it is believed to be an important regulator of several cell functions. Some of these functions have already been elucidated: For example, it has been shown that ATP signaling via $P2Y_{11}$ can delay the mitochondrial pathway of neutrophil apoptosis [160]. This receptor also seems to regulate activation, differentiation and other pro-inflammatory functions in macrophages [161]. Similarly, the release of pro-inflammatory cytokines like IFN-γ or IL-6 from keratinocytes was found to be influenced by $P2Y_{11}$ signaling [162], [163]. Regarding possible clinical implications, Amisten *et. al.* identified a small increase in the risk of acute myocardial infarction in study participants with an A87T polymorphism in the *P2RY11* gene, as this mutation is associated with increased systemic inflammation [164], [165]. Additionally, this receptor is believed to play a role in narcolepsy, as well as certain tumor types [166]–[168]. Although $P2Y_{11}$ receptors are among the purinergic receptors with the highest expression in T cells, little is known about their function in T cell activation [149].

The $P2Y_{11}$ receptor is among the least studied of the purinergic receptor family. Although some studies have identified potential functions, researching this receptor poses several

difficulties. First of all, there are very few inhibitors known to selectively block or stimulate $P2Y_{11}$ but none of the other purinergic receptors. Several researchers suggest using small interfering RNA (siRNA) for silencing this receptor, which is, however, very difficult to do in primary cells [169]. Another considerable obstacle in the research of $P2Y_{11}$ is the fact that no homologue of this receptor has been found in the genome of most rodents, which complicates the exploration of its functions in mouse models [169], [170]. Nonetheless, as mentioned above, the involvement of second messengers like Ca^{2+} and cAMP as downstream targets implies the functions of $P2Y_{11}$ to be significant.

The adenosine receptor A_{2A} also couples with G_s and utilizes cAMP as a downstream messenger, which leads to the hypothesis that it could have similar effects as $P2Y_{11}$. It has also been shown to be expressed highly on immune cells [171]. Although the activation of adenosine receptors is generally known for inducing anti-inflammatory pathways in humans, they could potentially have more diverse effects in mice due to the lack of murine $P2Y_{11}$ receptors [169]. While A_{2B} receptors work similarly to $A_{2A,}$ their abundance is a lot lower and they need considerably higher adenosine concentrations to be activated [91]. Investigating the correlation of the $P2Y_{11}$ and A_{2A} receptors in humans and mice could potentially facilitate the generation of mouse models and the further clinical investigation of the $P2Y_{11}$ receptor.

1.3 Aim of this thesis

T cell suppression in sepsis is a common phenomenon, although the underlying mechanisms remain unclear [172]. As described before, purinergic signaling is increasingly being recognized as a potent modulator of T cell activation and function. It has been shown that the disruption of this intricate feedback loop impairs T cell vigilance and the ability to properly become activated, which ultimately means a decreased immunopotency [173]. This could be the case in trauma and sepsis patients, and the ability to reverse those effects could potentially diminish the dangers and complications of sepsis [16]. This thesis aims to investigate the effects of $P2Y_{11}$ signaling on the formation and maturation of the immune synapse, as well as pivotal T cell functions like migration and proliferation. In detail, I want to find answers to the following questions:

How is $P2Y_{11}$ signaling involved in T cell activation/function?

- Does $P2Y_{11}$ influence T cell migration?
- Does $P2Y_{11}$ mediate immune synapse formation, and if yes, how?
    - Does $P2Y_{11}$ influence the binding capacity of T cells with APCs?
    - Does $P2Y_{11}$ influence mitochondrial translocation to the IS?
    - Does $P2Y_{11}$ influence mitochondrial activity?
- Does $P2Y_{11}$ alter Ca^{2+} signaling?
- Does $P2Y_{11}$ influence the functional T cell response?

This project is based on the hypothesis that $P2Y_{11}$ signaling, likely via cAMP, regulates the translocation of mitochondria along the microtubules to the IS, where they can provide the ATP necessary for the signal amplification loop. When this system is derailed, for example through high amounts of extracellular ATP, the mitochondria cannot adequately move towards the synapse, which disrupts the correct formation of the IS and ultimately the immune response.

2 Materials and Methods

2.1 Reagents

The essential activators and inhibitors used in this project are depicted in **Table 1**, along with their used concentrations, functions and sources. Unless otherwise specified, all other reagents were purchased from Sigma Aldrich (St. Louis, MO).

Table 1: Activators and inhibitors used. The following drugs were used during this project to study different pathways and modes of action. Most importantly, the $P2Y_{11}$ agonist (NF546) and antagonist (NF340) were used in all experiments.

Inhibitor	Concentration	Function	Source
NF340	20 µM	$P2Y_{11}$ receptor inhibitor	R&D Systems, Minneapolis, MN
NF546	20 µM	$P2Y_{11}$ receptor agonist	R&D Systems
H89	10 µM	protein kinase inhibitor (primarily PKA)	R&D Systems
cAMP-AM	10 µM	membrane-permeant precursor of cAMP	BIOLOG Life Science Institute, Bremen, Germany
Nocodazole	5 µM	microtubule polymerization inhibitor	R&D Systems
Latrunculin B	10 µM	actin polymerization inhibitor	Calbiochem, San Diego, CA
CSC	1 µM	A_{2A} receptor inhibitor	R&D Systems
CGS21680	100 nM	A_{2A} receptor agonist	R&D Systems

2.2 Cell culture

Jurkat T cells (acute T cell leukemia, clone E6-1) and U937 monocyte-like cells (histiocytic lymphoma) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI-1640 medium (ATCC), supplemented with 10% heat-inactivated fetal calf serum (FCS, Atlanta Biologicals, Flowery Branch, GA), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2. This medium is in the following referred to as "complete RPMI". Cell lines were used for experiments until passage 16.

2.2.1 PBMC isolation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the blood of healthy donors via the Ficoll separation technique (GE Healthcare, Chicago, IL). Briefly, a 1:1 mixture of blood and serum-free RPMI-1640 medium was layered onto 10 ml of Ficoll Paque solution and centrifuged for 30 min at 400 *g*. The uppermost layer containing serum and

medium was discarded. The mononuclear cell layer was transferred into a fresh tube and washed two times with warm RPMI-1640. PBMCs were counted and used for experiments immediately. All healthy donors gave written informed consent before the blood draw, and approval for studies involving human subjects was obtained from the Institutional Review Board of Beth Israel Deaconess Medical Center.

2.2.2 CD4+ T cell isolation

For positive selection of CD4+ T cells by MACS® Technology (Miltenyi Biotec, San Diego, CA), freshly isolated PBMCs were resuspended in cold phosphate buffered saline (PBS, GE Healthcare) supplemented with 0.5% bovine serum albumin (BSA) and incubated with anti-human CD4-labelled magnetic microbeads (Miltenyi Biotec) for 15 min at 4°C. Cells were then washed with PBS + 0.5% BSA at 400 *g* for 10 min. Subsequently, the cell suspension was placed into a magnetized MS column (MiniMACS Separator, Miltenyi Biotec). The column was washed three times with PBS + 0.5% BSA to remove all unbound cells. Then the magnet was removed and the CD4+ cells were eluted in PBS + 0.5% BSA. For further use, CD4+ T cells were resuspended in complete RPMI.

2.2.3 Plasmid transfection and silencing

Plasmids containing cDNA of the wild-type P2X4 receptor were obtained from Origene™ Technologies (Rockville, MD). Enhanced green fluorescent protein (EGFP)–tagged P2X4 receptor constructs were generated by ligating the receptor constructs with a pEGFP-N1 vector (Clontech Laboratories, Mountain View, CA) as previously established in the laboratory [144]. Transfections were performed using the Neon® Transfection system with 100 µl electroporation tips (MPK10096, Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. Approximately 3 to 3.5 million Jurkat cells were washed two times with serum-free RPMI-1640 and then resuspended in 100 µl of the hypo-osmolar electroporation buffer containing 10 µg of the EGFP-P2X4 plasmid. Electroporation was performed with 1700 V for 20 ms and cells were immediately transferred into antibiotics-free RPMI-1640 medium with 10% FCS. Imaging was performed after 4-6 h incubation at 37°C with 5% CO_2. To assess the distribution of the $P2Y_{11}$ receptor, Jurkat cells were transfected with 10 µg $P2Y_{11}$-YFP as described above.

A siRNA construct targeting $P2Y_{11}$ receptors (SMARTpool siRNA) was purchased from Dharmacon (Lafayette, CO). A non-targeting siRNA (Qiagen, Germantown, MD) was included as a negative control in all experiments. Approximately 3 to 3.5 million Jurkat cells were transfected with 10 nM of the respective siRNAs using the Neon® Transfection system as described for the plasmid transfection. The cells were cultured for 48 h before use, according to the previous gene knock-down optimization by the laboratory [144].

2.3 T cell motility

T cell motility and migration are essential tools in the immune response. To investigate whether the $P2Y_{11}$ receptor has any effect on this function, the motility of stimulated T cells was measured under the influence of several inhibitors. Therefore purified CD4+ T cells were resuspended in serum-free RPMI, which enhances adherence. They were then placed into fibronectin-coated (40 µg/ml) 8-well glass bottom chamber slides (Lab-Tek, Rochester, NY) and were allowed to adhere in the incubator (37°C and 5% CO_2) for 30 min. After two wash steps with complete RPMI the chambers were placed in a temperature controlled (37°C) stage incubator and maintained in a humidified gas atmosphere at 5% CO_2 and 21% O_2 (Live Cell Instrument, Seoul, Korea). T cells were treated with medium or inhibitors at the indicated concentrations 10 min prior to imaging. Migration was tracked in the presence or absence of 100 ng/ml SDF-1α (R&D Systems) as a stimulant by time-lapse microscopy with a Leica DMRI inverted microscope through a 20x objective (numerical aperture, NA 0.4; Leica Microsystems, Wetzlar, Germany) capturing 41 sequential images at 45 s intervals (30 min in total). The migration paths of individual cells were determined with ImageJ software (NIH; MTrackJ plugin) and used to calculate the migration speed.

2.4 Mitochondrial imaging

The mitochondria are indispensable organelles in the setting of the immune synapse because they provide the ATP for many energy-demanding processes during IS formation. In order to investigate the effect of $P2Y_{11}$ on the mitochondria, I tested not only their activity upon the addition of inhibitors of interest, but also their translocation towards the synapse and their colocalization with the P2X4 receptor upon stimulation.

2.4.1 Mitochondrial activity

For the analysis of mitochondrial activity with and without stimulation, Jurkat and U937 cells were stained separately with 100 nM MitoTracker Red CM-H2Xros or MitoTracker Green (both from Thermo Fisher Scientific), respectively, for 10 min at 37°C. After two wash steps with serum-free RPMI-1640 to enhance adherence, the cell lines were mixed in a 1:1 ratio and then placed into fibronectin-coated (40 µg/ml) 8-well glass bottom chamber slides (approx. $2.5*10^5$ per cell type per well). They were allowed to settle and adhere in the incubator for 20-30 min before undergoing 3 wash steps with complete RPMI. Fluorescence live-cell imaging was performed with an inverted Leica DMI6000B microscope (Leica Microsystems) equipped with a temperature-controlled (37°C) stage incubator and a Leica DFC365 FX camera. Images and movies were captured through a 100x objective (NA 1.4, immersion oil) using fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) filter sets (Leica Microsystems) and the LeicaLAS microscope imaging software (version 2.0). Time-lapse imaging of the cells was performed for 5 min using 5 s intervals. Complete RPMI or inhibitors at the indicated concentrations were added

after 15 s (frame 3) of the video. The fluorescence intensity of the ROS-sensitive MitoTracker Red CM-H2Xros was measured for each Jurkat cell over time using the ImageJ software. This was used to calculate changes in mitochondrial activity upon the addition of medium or inhibitors.

2.4.2 Mitochondrial translocation to the synapse

In order to quantify the mitochondrial translocation to the immune synapse under influence of the indicated inhibitors, Jurkat and U937 cells were stained with 100 nM MitoTracker Red CM-H2Xros or MitoTracker Green, respectively, at 37°C for 10 min. After two wash steps with serum-free RPMI both cell types were mixed at a 1:1 ratio and placed into fibronectin-coated (40 µg/ml) 8-well glass bottom chamber slides (approx. $2.5*10^5$ per cell type per well) in the incubator for 20-30 min to settle and adhere. Unbound cells were washed away with complete RPMI before imaging. Similarly to the activity measurements, cells were first treated with complete RPMI or inhibitors at the indicated concentrations for 10 min prior to the addition of 0.5 µg/ml anti-human CD3 antibody (BD Pharmingen, San Jose, CA) to stimulate synapse formation. Fluorescence live-cell imaging was performed using an inverted Leica DMI6000B microscope with a temperature-controlled (37°C) stage incubator and a Leica DFC365 FX camera. Images were taken through a 100x objective (NA 1.4, immersion oil) using FITC and TRITC filters and LeicaLAS imaging software. To assess mitochondrial location within the cells, overlays of bright field and fluorescent images were analyzed with ImageJ. Gray values of the MitoTracker Red CM-H2Xros fluorescence were measured separately in the leading edge and the uropod of Jurkat cells that had formed a synapse with U937 cells. The mitochondrial translocation was then calculated as a ratio between the mean fluorescence values of the leading edge and the uropod.

For 3D imaging of the synapse, Jurkat cells were stained with 100 nM Mitotracker Red CM-H2Xros before mixing them at a 1:1 ratio with unstained U937 cells and washing the mixture two times with Hank's balanced salt solution (HBSS, GE Healthcare). All cells were then transferred into fibronectin-coated (40 µg/ml) 8-well glass bottom chamber slides and incubated for 30 min to settle and adhere. After two wash steps with HBSS, cells were stained with 500 nM of a cell surface-targeting fluorescent ATP probe (2-2Zn, gift from Itaru Hamachi, Kyoto University, Kyoto, Japan) in the incubator for 10 minutes. Without washing, cells were then treated with HBSS or inhibitors for 10 min before synapse formation was induced by adding 0.5 µg/ml anti-human CD3 antibody. Imaging was performed with a SpinSR10 spinning-disc confocal microscope (Olympus Lifescience, Tokyo, Japan) using a 100x objective (NA 1.49, immersion oil) and a temperature-controlled (37°C) stage incubator. Using an ORCA-Flash4.0 V3 Digital CMOS camera (Hamamatsu Photonics, Hamamatsu, Japan) and FITC/TRITC filters, Z-stacks of synapse-forming cells were acquired for 5 min. Maximum projections of the Z-stacks were created using the ImageJ

software and are displayed as qualitative data to accurately depict 3D localization of mitochondria within synaptic T cells.

2.4.3 Colocalization of mitochondria and P2X4

To test the hypothesis that the $P2Y_{11}$ receptor modulates the colocalization of P2X4 and mitochondria in activated T cells, Jurkat cells were transfected with an EGFP-tagged P2X4 receptor plasmid as described above. After 4-6 h of incubation in antibiotics-free medium, cells were resuspended in complete RPMI and stained with 100 nM Mitotracker Red CM-H2Xros for 10 min at 37°C. After two wash-steps with serum-free RPMI-1640, cells were transferred into fibronectin-coated (40 µg/ml) 8-well glass bottom chamber slides and incubated for 30 min to settle and adhere (approx. $5*10^5$ per well). Prior to imaging, cells were treated either with medium or the indicated inhibitors for 10 min before adding 100 ng/ml of SDF-1α in order to stimulate colocalization. Fluorescence live-cell imaging was performed using the inverted Leica DMI6000B microscope with a temperature-controlled (37°C) stage incubator and a Leica DFC365 FX camera as described above. Images were taken through a 100x objective (NA 1.4, immersion oil) using FITC and TRITC filters. Overlays of bright field and fluorescence images were generated to assess the colocalization of mitochondria and P2X4 receptors within stimulated cells. These images are depicted as qualitative data.

2.5 Immune synapse formation

The formation of immune synapses between T cells and APCs represents a crucial part of a functional immune response, since it ensures proper antigen presentation. Therefore I established two methods to see whether the $P2Y_{11}$ receptor or other purinergic components had an effect on immune synapse formation in Jurkat cells or primary PBMCs.

2.5.1 Jurkat cells

Unstained Jurkat cells (approx. $5*10^5$/ml) were transferred into a flat-bottom 96-well plate and treated with medium or inhibitors at the indicated concentrations in the incubator for 10 min. Cells were subsequently stimulated to form synapses with anti-CD3/anti-CD28 antibody-coated magnetic Dynabeads (Invitrogen Dynal AS, Oslo, Norway) in a 1:1 cell-to-bead ratio for 15 minutes at 37°C. They were then measured by flow cytometry using the BD FACSCalibur Cell Analyzer (Becton Dickinson, San Jose, CA) and the BD Cellquest™ software (Becton Dickinson) with settings displayed in **Table 2**.

Table 2: Flow cytometry acquisition settings for synapse formation measurements with Jurkat cells and anti-CD3/anti-CD28 magnetic beads.

Detector	Voltage	AmpGain	Mode
FSC	E00	1.00	Lin
SSC	370	1.00	Lin
FL3	650	1.00	Log

All samples were analyzed according to their forward/side scatterplots using the FlowJo® software (Becton Dickinson). The population that only occurred in samples incubated with beads was analyzed and compared among treatments. The percentage of this population was calculated in comparison to the unbound Jurkat cells. Unbound beads were not included in the calculation.

2.5.2 PBMCs

For the identification of synaptic cells, PBMCs were stained with 0.25 µg/ml allophycocyanin-conjugated anti-CD4 and 2 µg/ml AlexaFluor®488-conjugated anti-CD11b antibodies (both from Biolegend, San Diego, CA) for 20 min in the incubator. They were then transferred into a flat-bottom 96-well plate ($1*10^6$/ml) and then incubated with complete RPMI or inhibitors for 10 min at 37°C. Subsequently, cells were stimulated with 0.5 µg/ml anti-CD3 antibody for 1 h in the incubator. Samples were carefully transferred into flow cytometer measuring tubes, in order to avoid breaking up the synapses. They were then measured with the BD FACSCalibur Cell Analyzer with the settings shown in **Table 3**.

Table 3: Flow cytometry acquisition settings for synapse formation measurements with anti-CD3 antibody-stimulated PBMCs.

Detector	Voltage	AmpGain	Mode
FSC	E00	1.50	Lin
SSC	355	1.00	Lin
FL1	670	1.00	Log
FL4	750	-	Log

Using the FlowJo® software, CD4+ and CD11b+ cells were identified and all double-positive events were assumed as synapses between monocytes and CD4+ T cells. When analyzing the double-positive events in the forward/side scatterplot, this population was observed to be located far right in the forward scatter (see flow cytometry panels in the Results section). For subsequent experiments this population was analyzed without staining to avoid possible interference of the antibodies with the synapse formation.

2.6 Functional assays

In order to investigate the influence of $P2Y_{11}$ on T cells, the effects on T cell function had to be measured as well. Therefore one early and one long-term parameter of T cell activation were chosen.

2.6.1 Calcium signaling

Upon stimulation, T cells show a characteristic augmentation in their intracellular Ca^{2+} signaling. To quantify the effects of purinergic components on this well-known phenomenon, PBMCs were resuspended in HBSS and stained with 4 µM of the cytosolic calcium indicator Fluo-4 (Thermo Fisher Scientific) and 0.25 µg/ml APC-conjugated anti-CD4 antibody in the incubator for 20 min. After two wash steps with HBSS, the cells were transferred into fibronectin-coated (40 µg/ml) 8-well glass bottom chamber slides ($1.5*10^6$/ml) and placed into the incubator for 30 min to settle and adhere. They were washed once with HBSS prior to imaging with an inverted Leica DMI6000B microscope equipped with a temperature-controlled (37°C) stage incubator. FITC and deep-red Cy5 filters were used. Bright field image overlays with the Cy5 fluorescence images were used to identify CD4+ T cells. For Ca^{2+} signal analysis over time, only the FITC filter was applied and images were taken in 3 s intervals for 5 min. After 45 seconds (frame 15), either HBSS or the indicated inhibitors were added. In a second movie 0.1 µg/ml mouse anti-human CD3 and CD28 antibodies (BD Pharmingen) were added and crosslinked with 0.1 µg/ml anti-mouse IgG (Thermo Fisher Scientific) after 45 seconds. Bright field, Cy5 and FITC images were taken after each respective movie as well. Using the ImageJ software, CD4+ cells were identified in the pre-movie images and mean Fluo-4 fluorescence was measured over time in the selected cells. To compare different experiments all fluorescence values were normalized to the first frame and expressed as fold-change. Additionally, the peak intensity was compared among treatments by selecting a time frame of 15 s around the highest value of the control sample.

2.6.2 Proliferation

To measure proliferation, a key function of activated T cells, PBMCs were stained with 5 µM carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher Scientific) for 5 min at room temperature in the dark. After three wash steps with complete RPMI, cells were transferred to a flat-bottom 96-well plate (approx. $8*10^5$/ml) and treated with medium or inhibitors at 37°C and 5% CO_2 for 15 min. Cells were subsequently stimulated with 0.1 µg/ml anti-CD3 antibody. After 72 h incubation cells were spun down at 400 *g* for 10 min and stained with 0.25 µg/ml anti-CD4 antibody for 20 min at 4°C. After diluting the samples 1:5 in HBSS they were measured with the BD FACSCalibur Cell Analyzer with the settings shown in **Table 4.**

Table 4: Flow cytometry acquisition settings for CFSE dilution/proliferation measurements with anti-CD3 antibody-stimulated PBMCs.

Detector	Voltage	AmpGain	Mode
FSC	E00	1.50	Lin
SSC	380	1.00	Lin
FL1	620	1.00	Log
FL4	750	-	Log

2.7 Statistics

All statistical analyses were performed using the GraphPad Prism software version 5. Unless otherwise stated, data are expressed as mean values ± standard error (SE) of $n \geq 3$ independent experiments. Two-tailed unpaired Student's *t* test or one-way ANOVA and Dunett's Multiple Comparison Test (vs. control) were used to compare differences between two or multiple groups, respectively. Differences were considered significant at $p < 0.05$.

3 Results

3.1 Autocrine stimulation of $P2Y_{11}$ receptors is necessary for T cell migration

Purinergic signaling has repeatedly been shown to play a role in the migration of immune cells like neutrophils and macrophages [78], [127], [137]. Recently, specific purinergic components such as the P2X4 receptor have been linked to T cell migration as well [145]. Since the $P2Y_{11}$ receptor is, together with the P2X4 receptor, amongst the most highly expressed purinergic receptors in T cells, the logical step was to investigate if there is a connection between the two, specifically in the setting of T cell motility and migration [149]. Therefore, the effects of $P2Y_{11}$ modulators on the motility of stimulated Jurkat and primary CD4+ T cells were tested. Stimulation of T cells with the chemokine SDF-1α characteristically leads to a substantial increase in motility. Pre-treatment of CD4+ T cells with both the $P2Y_{11}$ agonist and antagonist significantly reduces this effect (**Figure 8A**). Unexpectedly, the addition of the $P2Y_{11}$ agonist leads to even lower cell movement than the inhibitor, although it has previously been shown to positively stimulate the receptor [169], [174]. In **Figure 8B** CD4+ T cells were stimulated with beads carrying anti-CD3 and anti-CD28 antibodies for 3 days to induce highly motile lymphoblasts. Similarly to the stimulation with SDF-1α, these T cells are significantly less motile when treated with the $P2Y_{11}$ agonist or inhibitor. An important molecule downstream of $P2Y_{11}$ is cAMP, the addition of which also causes a significant decrease in T cell motility. However, the PKA inhibitor H89 surprisingly reduces T cell motility as well, even though the cAMP-dependent PKA is known to suppress T cell activation. Together, these findings suggest that T cell motility upon stimulation is at least partially dependent on functional $P2Y_{11}$ signaling.

The lower panel of **Figure 8** shows similar effects in Jurkat cells, a T cell line that is often used to study CD4+ T cell activation mechanisms and that also reacts to SDF-1α stimulation. Unstimulated Jurkat cells show a moderate spontaneous motility, which is suppressed after $P2Y_{11}$ agonist or inhibitor treatment. An even higher inhibition can be achieved in Jurkat cells stimulated with SDF-1α after pre-treatments with the indicated inhibitors (**Figure 8C**). Interestingly, also cAMP and H89 suppress motility in both unstimulated and stimulated Jurkat cells, suggesting that they regulate motility in a TCR signaling-independent fashion. In the lower right panel of the figure, Jurkat cells were transfected with $P2Y_{11}$ siRNA. Silencing of the $P2Y_{11}$ receptor leads to a significant reduction of motility in both unstimulated and SDF-1α-stimulated cells (**Figure 8D**). This indicates again that, similarly to primary CD4+ T cells, $P2Y_{11}$ signaling is a regulator of T cell motility and migration in Jurkat cells. Subsequently, the question how $P2Y_{11}$ and P2X4 receptor-dependent pathways are connected to each other to regulate T cell migration had to be addressed.

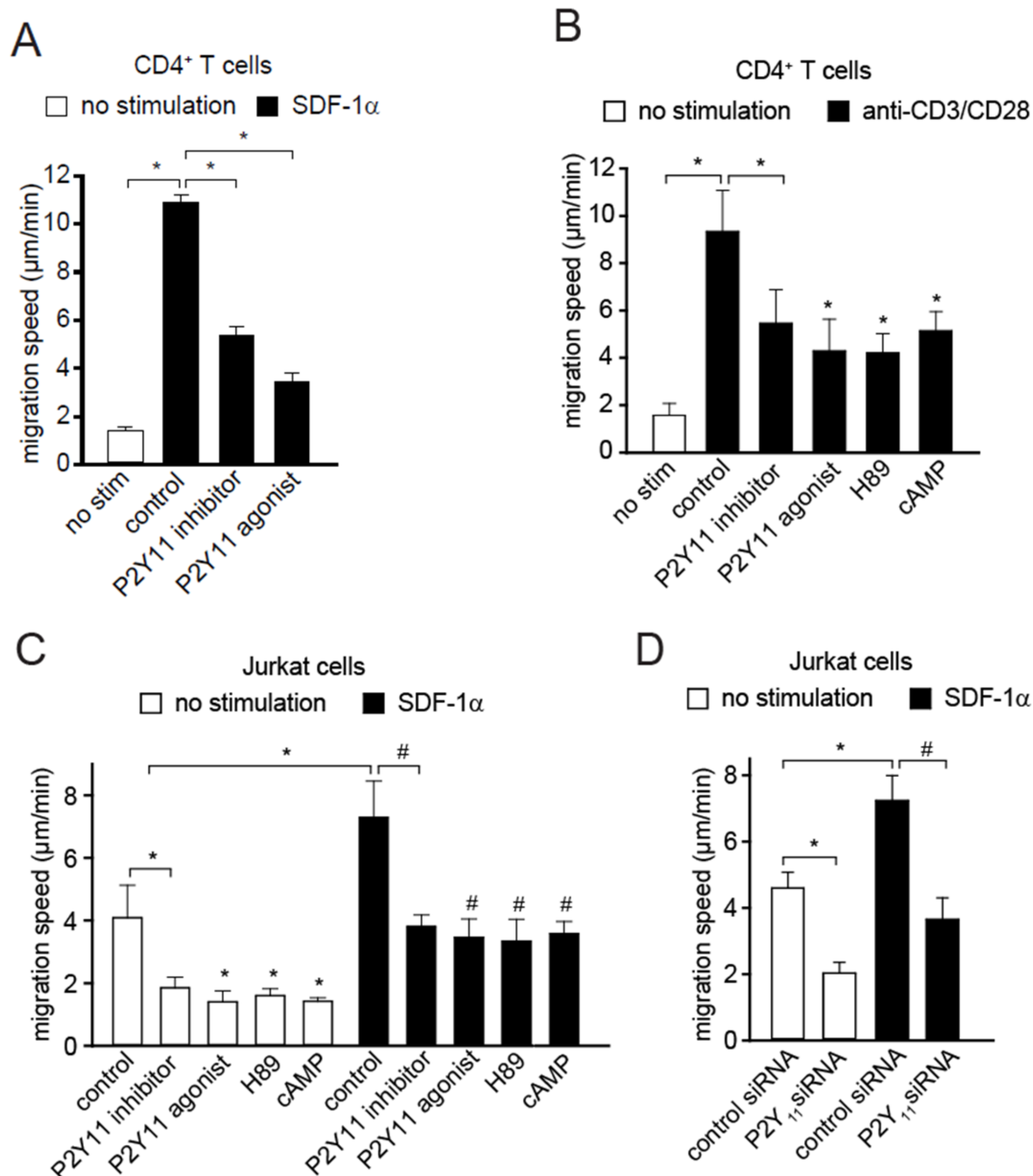


Figure 8: P2Y$_{11}$ receptors facilitate T cell motility. Migration of T cells was tracked over a time period of 30 min by time-lapse microscopy and migration speed of single T cells was determined using ImageJ. (A) Freshly isolated CD4+ T cells were pre-treated with medium or the P2Y$_{11}$ agonist or inhibitor (both 20 µM) before stimulation with 100 ng/ml SDF-1α. (B) CD4+ T cells were stimulated for 3 days with anti-CD3/CD28 antibody-coated beads and migration was analyzed in the presence or absence of the P2Y$_{11}$ agonist (20 µM), antagonist (20 µM), H89 (10 µM) or cAMP-AM (10 µM). (C) Jurkat cells were pre-treated with P2Y$_{11}$ modulators (both 20 µM), H89 (10 µM) or cAMP-AM (10 µM) and migration was measured in the presence or absence of SDF-1α. (D) Jurkat cells were transfected with non-targeting or P2Y$_{11}$ siRNA before measuring migration in the presence or absence of SDF-1α. Data represented as mean ± SD of 3 independent experiments, each comprising at least 40 cells. $^{*,\#}P < 0.05$ vs. control (one-way ANOVA).

3.2 $P2Y_{11}$ regulates the colocalization of mitochondria and P2X4 in migrating T cells, but not mitochondrial activity

It has been shown that purinergic P2X4 receptors tend to accumulate together with the mitochondria at the leading edge of stimulated T cells [144], [175], [176]. However, it has never been tested if these processes happen in a coordinated fashion or completely independently of each other. The fact that mitochondria produce ATP which can be released to stimulate P2X4 receptors suggests that there is, in fact, a connection. In contrast to the P2X4 receptor, $P2Y_{11}$ does not accumulate at the leading edge of stimulated T cells, but moves to the uropod instead (**Figure 9B**). Since $P2Y_{11}$ seems to work closely together with P2X4 regarding migration, it might also play a role in the colocalization of P2X4 with mitochondria at the leading edge. To test this theory, Jurkat cells were transfected with EGFP-tagged P2X4 and stained them with a MitoTracker. **Figure 9A** shows that both components accumulate together at the leading edge of stimulated cells, while the addition of the $P2Y_{11}$ inhibitor/agonist and H89 prevents this phenomenon. This suggests that $P2Y_{11}$ signaling might influence the translocation of P2X4 to the leading edge of stimulated T cells and therefore indirectly facilitates migration.

Meanwhile it had to be tested if $P2Y_{11}$ had an impact not only on the location but also on the activity of mitochondria. Jurkat cells were stained with the ROS-sensitive MitoTracker Red CM-H2Xros and its fluorescence intensity was measured in the microscope. Neither $P2Y_{11}$ modulators nor H89 or cAMP have a visible effect on the activation of mitochondria, as can be seen in **Figure 9C**. Similarly, the agonist and inhibitor of the A_{2A} receptor, which is a $G\alpha_s$ coupled receptor and increases intracellular cAMP, similar to $P2Y_{11}$, does not alter mitochondrial activity either. **Figure 9C** and **D** show that only nocodazole, an inhibitor of microtubule polymerization, is able to significantly reduce mitochondrial activity. These findings indicate that $P2Y_{11}$ works by controlling the location of mitochondria and other components, rather than by modulating the organelle function directly. More evidence for this theory will also be shown later on.

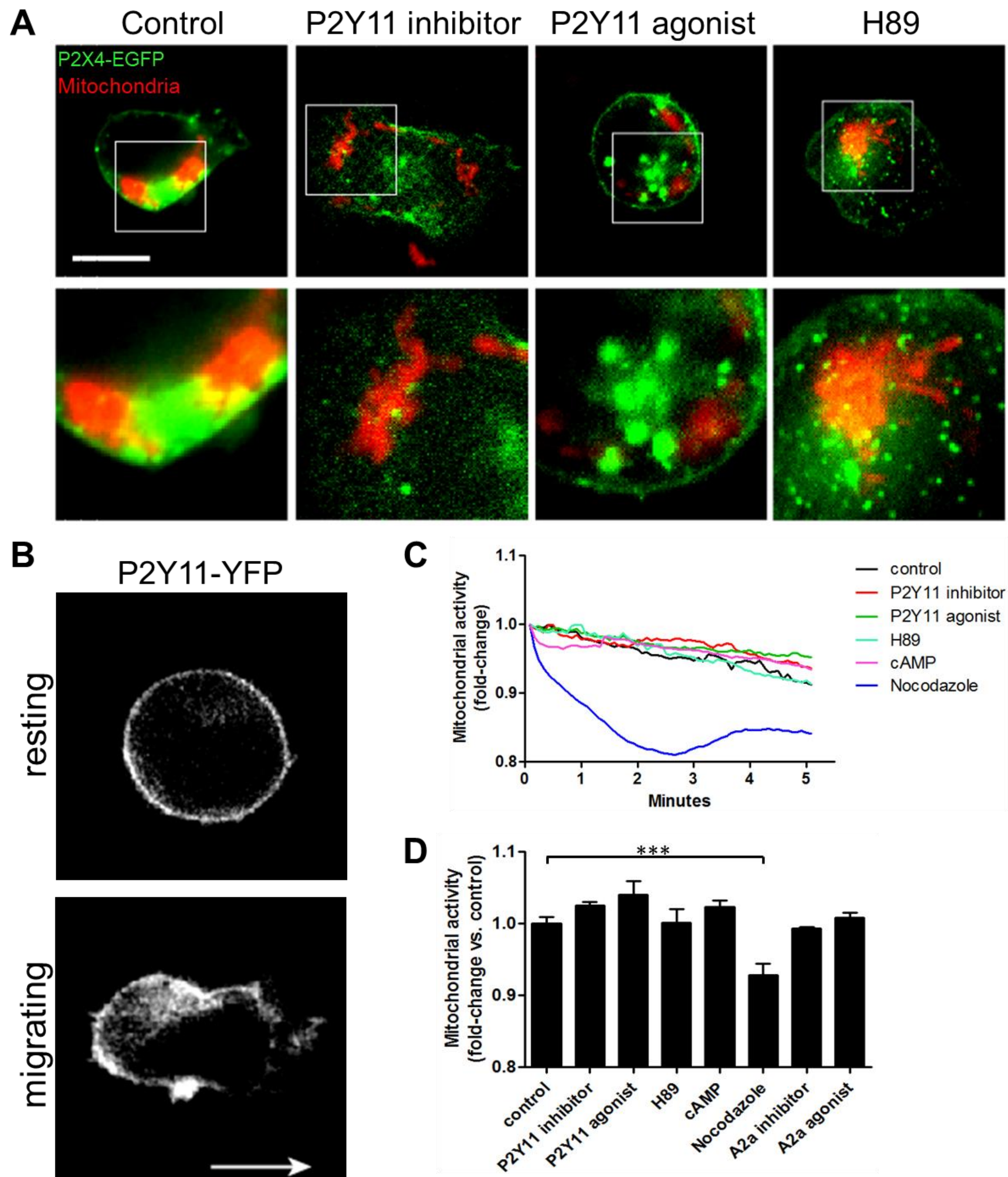


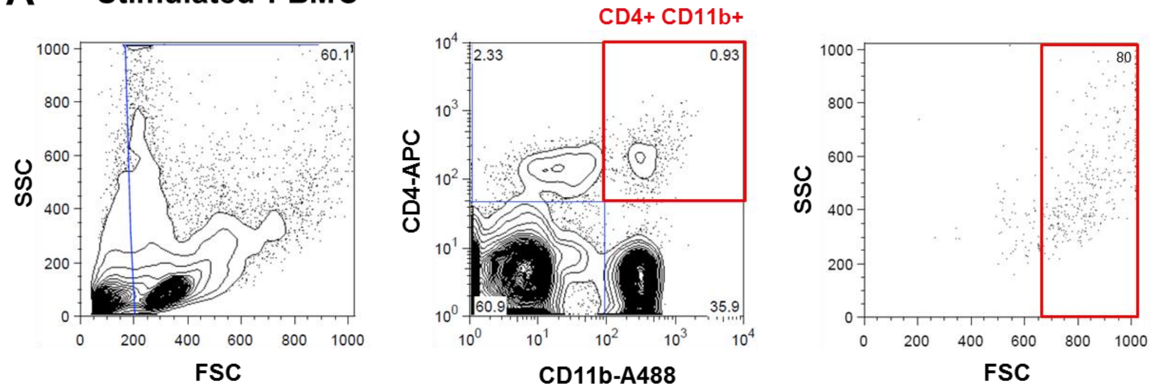
Figure 9: $P2Y_{11}$ facilitates the colocalization of P2X4 receptors and mitochondria. (A) Fluorescence overlays of P2X4-EGFP-transfected Jurkat cells stained with MitoTracker Red CM-H2Xros. Cells were pre-treated with medium, P2Y11 inhibitor or agonist (20 µM), or H89 (10 µM) before stimulation with 100 ng/ml SDF-1α. Images were obtained with a 100x objective. (B) Jurkat cells were transfected with $P2Y_{11}$-YFP before stimulation with SDF-1α and images of resting or migrating cells were taken with a 100x objective. (C) Time lapse analysis of mitochondrial activity in unstimulated Jurkat cells after the addition of medium, the $P2Y_{11}$ inhibitor or agonist (20 µM), H89 (10 µM), cAMP (10 µM), the A_{2a} inhibitor (1 µM) or agonist (100 nM), or nocodazole (5 µM). (D) Statistical analysis of the endpoint in (C). *$P < 0.05$ vs. control (one-way ANOVA).

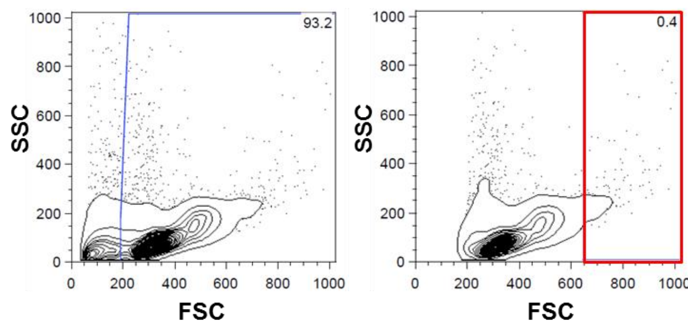
3.3 Initial immune synapse formation is independent of $P2Y_{11}$ signaling

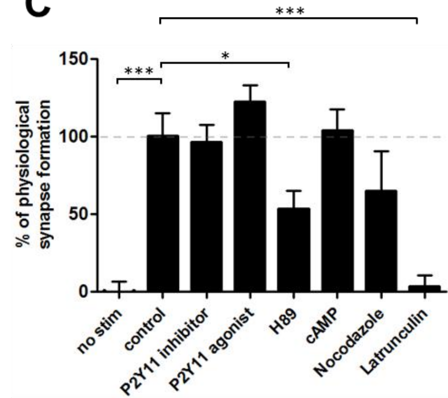
Upon contact, T cells and APCs form a transient but strong connection around their TCR-MHC complexes. This immune synapse is essential to ensure proper TCR and downstream signaling, as well as the exchange of messengers like ATP between the cells. Alongside the TCR and MHC, countless more molecules are involved in the formation of this synapse, including several co-receptors as well as actin and integrins. The preliminary hypothesis claimed that $P2Y_{11}$ signaling also plays a role in this complex mechanism. To test this hypothesis, I developed a method to quantify the formation of cell synapses (**Figure 10**). As described before, PBMCs were stained with fluorochrome-conjugated anti-CD4 and anti-CD11b antibodies. Then they were stimulated with anti-CD3 antibody for 60 minutes and measured by flow cytometry. The gating was performed as follows: First, thrombocytes and residual erythrocytes were excluded in the forward/side scatterplot to avoid interference with the relative cell numbers (**Figure 10A, left**). Then, using the allophycocyanin and Alex®Fluor-488 channels, CD4+ and CD11b+ double positive events were identified (**Figure 10A middle**). These double stained events were regarded as synapses between T cells and monocytes. This population is then again shown in the forward/side scatterplot to identify where the synaptic cells would show up. In the right panel of **Figure 10A** it can be seen that they accumulate in the far right of the forward/side scatterplot. For the following experiments the staining was omitted in order to avoid the possibility of interference of the antibodies with the synapse formation. **Figure 10B** shows the gating strategy for the unstained stimulated PBMCs in the forward/side scatterplot. Again, the thrombo- and erythrocytes were excluded and the far right events were considered as synapses. **Figure 10C** depicts the statistical analysis of 4 experiments (1 stained, 3 unstained) where PBMCs were pre-treated with the indicated inhibitors prior to stimulation. In contrast to our expectations, neither the $P2Y_{11}$ agonist nor the antagonist significantly alter immune synapse formation, although there is a trend that the agonist might actually cause an increase. H89 causes a modest decrease in synapse formation, while cAMP treatment does not lead to significant changes. Regarding cytoskeletal proteins, only actin seems to play a pivotal role in the initial synapse formation, since latrunculin almost completely hampers synapse formation. Meanwhile the microtubule inhibitor nocodazole does not have a significant effect, although a slight downward trend can be observed.

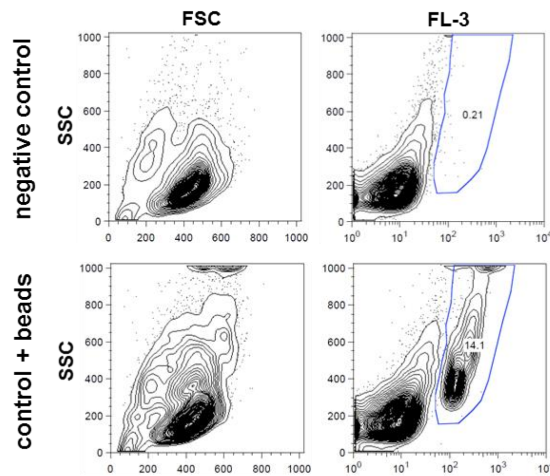
To investigate these results also in Jurkat cells, they were incubated with anti-CD3/CD28 antibody-coated magnetic beads. Since these beads exhibit a minor intrinsic fluorescence, it was possible to identify the cells that had bound to beads without staining. While there are diffuse changes in the forward/side scatterplot, a clearly segregated population in the FL3 channel that does not show up in samples without beads is observable (**Figure 10D**). When testing whether $P2Y_{11}$ had an effect on this population by adding the agonist or inhibitor prior

to the addition of the beads, neither the agonist nor the antagonist of $P2Y_{11}$ alters the synapse formation in Jurkat cells (**Figure 10E**).









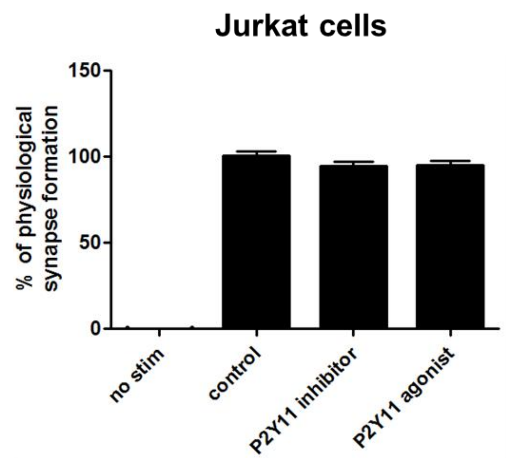


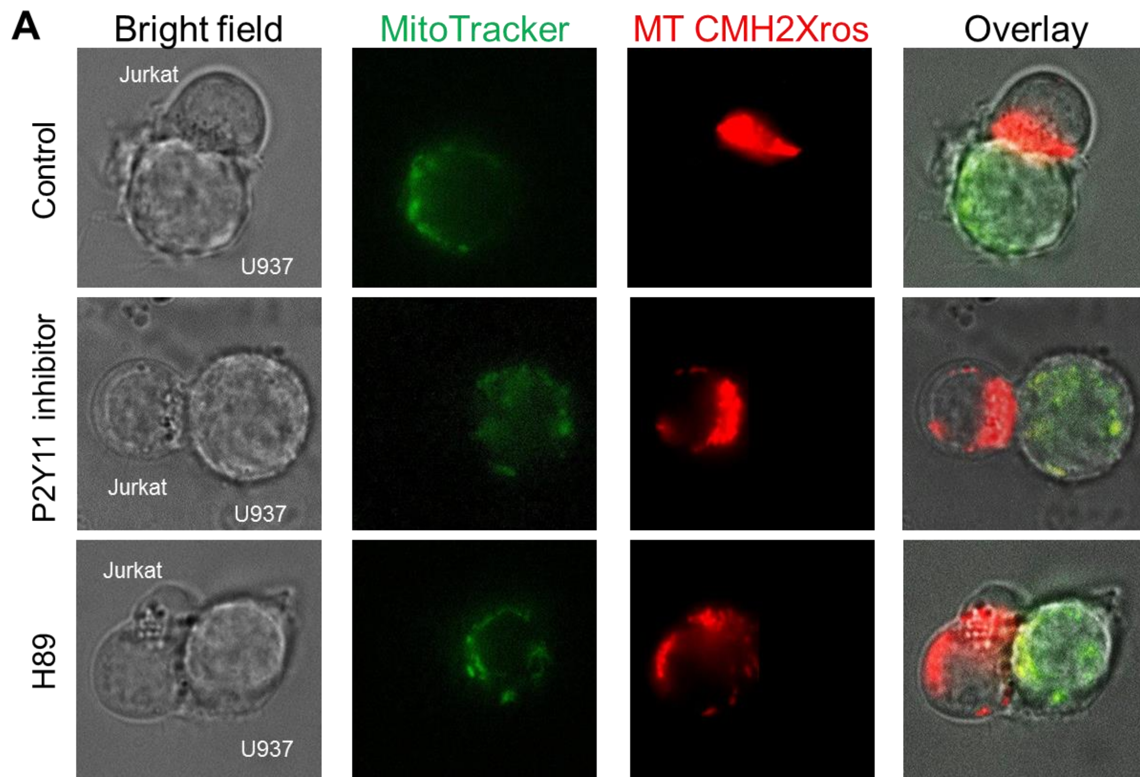
Figure 10: Immune synapse formation is not influenced by $P2Y_{11}$ signaling. (A) Gating strategy for PBMCs stained with anti-CD4-allophycocyanin and anti-CD11b-Alexa®Fluor-488. After excluding thrombocytes and residual erythrocytes, CD4+CD11b+ double-positive events were identified and observed in the forward/side scattergram. (B) Gating strategy for unstained PBMCs. After gating out thrombocytes and residual erythrocytes, the far right events were regarded as synaptic cells. (C) Statistical analysis of synapse formation in unstimulated or anti-CD3 antibody-stimulated PBMCs that were pre-treated either with medium or the $P2Y_{11}$ agonist/antagonist (20 µM), H89 or cAMP-AM (10 µM), nocodazole (5 µM), or latrunculin (10 µM). Data is presented as mean ± SE of 4 individual experiments (*$P < 0.05$ vs. control, one-way ANOVA). (D) Gating strategy for Jurkat cells stimulated with anti-CD3/CD28 antibody-coated beads. A characteristically segregated population appears in bead-stimulated Jurkat cells in the FL-3 channel. (E) Statistical analysis of synapse formation in unstimulated or bead-stimulated Jurkat cells that were pre-treated either with medium or the $P2Y_{11}$ agonist/antagonist (20 µM). Data from 6 individual experiments is displayed as % of the stimulated control (mean ± SE).

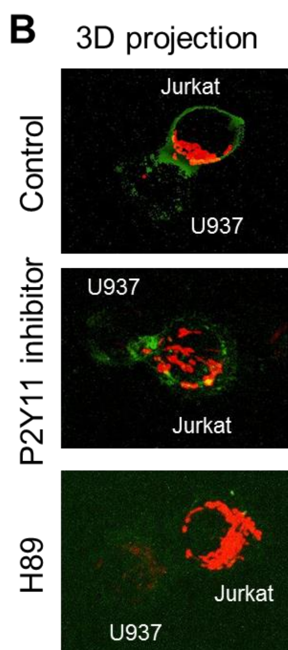
3.4 $P2Y_{11}$ guides mitochondrial translocation to the immune synapse

Since it was shown that $P2Y_{11}$ does not have an impact on the initial synapse formation, its effect was hypothesized to be further downstream in the maturation of the synapse. It is already established that this receptor influences the location of mitochondria in migrating cells, thus the next step was to investigate if it also regulated mitochondrial translocation to the immune synapse. Therefore, Jurkat and U937 cells were treated with the indicated inhibitors before stimulating synapse formation with anti-CD3 antibody. Images were taken after 10 minutes. As can be seen in **Figure 11A**, the mitochondria in the control Jurkat cells accumulate tightly at the synapse. Meanwhile, most mitochondria in the $P2Y_{11}$ inhibitor-treated cells tend to move toward the synapse, but do not go there completely. The translocation in H89-treated cells is reduced to less than 50%. This supports the theory that PKA might have some pro-inflammatory functions as well, since its inhibition hampers with these essential functions of the immune response in T cells. While the $P2Y_{11}$ agonist, cAMP, and the A_{2A} inhibitor and agonist affect mitochondrial translocation similarly to the $P2Y_{11}$ inhibitor, nocodazole suppresses it almost completely (**Figure 11C**). Interestingly, the location of mitochondria in the U937 cells is not visibly affected by stimulation or any of the inhibitors.

However, 2D microscopy only allows imaging of just one plane. So in order to validate these findings, 3D images of synaptic cells pre-treated with the above-mentioned inhibitors were obtained. **Figure 11B** shows the maximum projections (overlays of all Z stacks) of selected images. Identically to the 2D images, the mitochondria move completely to the synapse in control cells. The cells pre-treated with the $P2Y_{11}$ inhibitor clearly show a trend to move their mitochondria toward the synapse, but the process is immensely decelerated. H89

decreases the translocation even more, similar to the 2D images. Together, these findings imply that $P2Y_{11}$, while not affecting the initial formation of immune synapses, has a large impact on their maturation and functionality by mediating the translocation of mitochondria toward the location of high ATP-consumption. The A_{2A} receptor seems to have a similar effect, further strengthening the theory that they have correlated functions.





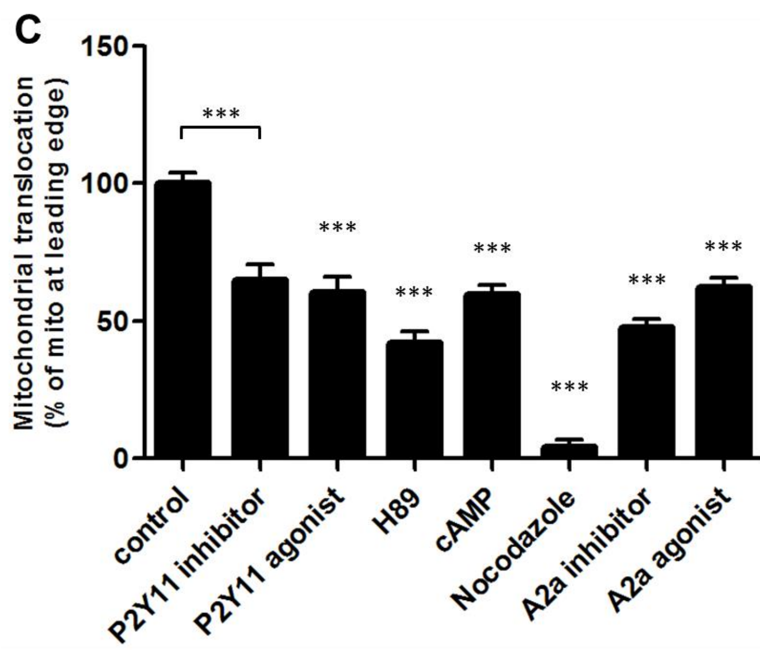


Figure 11: $P2Y_{11}$ regulates mitochondrial translocation to the synapse. (A) Jurkat and U937 cells were stained with MitoTracker CM-H2Xros or MitoTracker Green, respectively, before mixing and acquiring bright field and fluorescent images with a 100x objective. Synapse formation was induced by adding 0.5 µg/ml anti-CD3 antibody and mitochondrial location was assessed after 10 min. (B) Jurkat cells were stained with MitoTracker CM-H2Xros before mixing with U937 cells and staining with the membrane-bound ATP Probe 2-2Zn. Synapse formation was induced with anti-CD3 antibody and Z-stack images of whole cells were obtained with a 100x objective. This figure shows the maximum projection. (C) Statistical analysis of mitochondrial translocation, calculated as a ratio between mitochondria at the leading edge and the uropod and depicted as % of the control. Data from minimum 100 cells in 3 individual experiments is displayed as Mean ± SE (*$P < 0.05$ vs. control, one-way ANOVA).

3.5 $P2Y_{11}$ influences cytosolic Ca^{2+} signaling in stimulated CD4+ T cells

As described earlier, Ca^{2+} is an essential part of T cell activation by interacting with calcineurin and calmodulin and activating several pro-inflammatory transcription factors [29]. Upon stimulation, T cells characteristically show a rapid increase in cytosolic Ca^{2+} as a response to TCR signaling. Since $P2Y_{11}$ interferes with TCR signaling via cAMP, the components of the $P2Y_{11}$ pathway could potentially alter the Ca^{2+} response to stimulation. **Figure 12A** depicts the cytosolic Ca^{2+} signal over time, calculated in fold-change of the first frame. Notably, as can be seen in the left half of the graph, the inhibitors themselves have almost no effect on basal Ca^{2+} signaling in unstimulated T cells. This can also be observed in **Figure 12B**. Only cAMP (pink) causes a steady and significant rise in Ca^{2+} signaling. Although the PKA inhibitor H89 (turquois) seems to slightly elevate basal signaling, it does not make a significant difference compared to the control (black). While nocodazole (blue) initially seems to cause a drop in Ca^{2+} signal, it quickly recovers to basal control levels. The $P2Y_{11}$ inhibitor (red) as well as the agonist (green) do not change basal Ca^{2+} signaling. The right half of **Figure 12A** shows changes in Ca^{2+} signal of pre-treated T cells upon stimulation. The control cells show the expected peak approximately 2 min after activation. Cells pre-treated with the $P2Y_{11}$ inhibitor show an initial drop in Ca^{2+} signal, followed by a minimal increase. Compared to the control, the $P2Y_{11}$ agonist shows a peak similar in shape, but not in height, and there is also an initial drop in Ca^{2+} before it goes up. H89-treated cells even further decrease their Ca^{2+} signaling upon stimulation, while cAMP- and nocodazole-treated cells show only a slight increase. To compare the peak heights of the treatments, the fluorescence values of 15 seconds around the highest signal of the control cells were plotted for every treatment and calculated as fold-change of the control peak (**Figure 12C**). The $P2Y_{11}$ inhibitor as well as H89 reduce the physiological Ca^{2+} response to T cell activation significantly, to 0.73 and 0.6, respectively. Nocodazole and cAMP slightly decrease it as

well, although only nocodazole is significant (0.8). The $P2Y_{11}$ agonist does not significantly alter Ca^{2+} signaling, although a trend to decrease is clearly visible. In summary, the $P2Y_{11}$ receptor does not interfere with basal cytosolic Ca^{2+} signaling, but with the Ca^{2+} response to T cell stimulation.

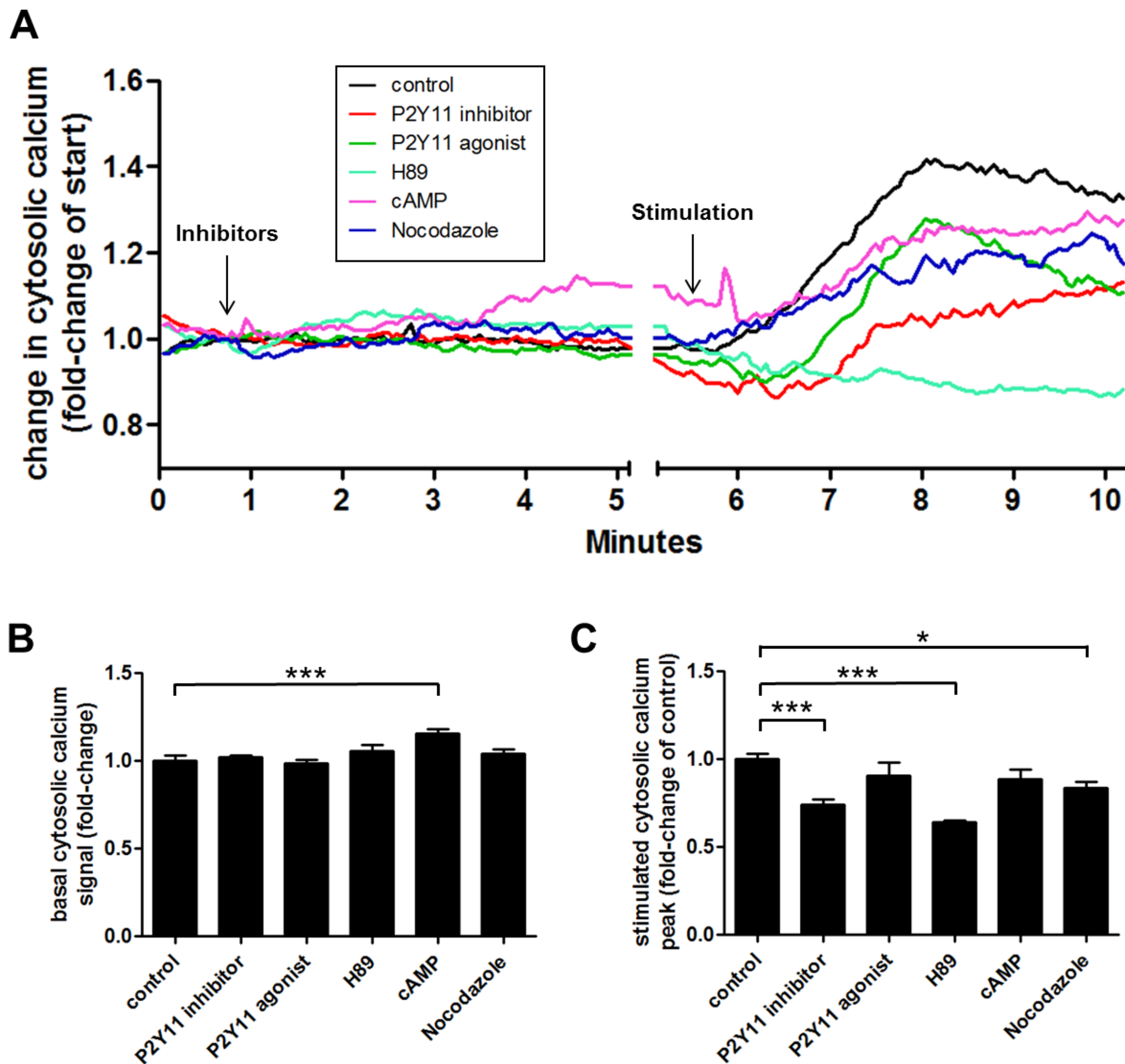


Figure 12: $P2Y_{11}$ influences basal and stimulated Ca^{2+} signaling of CD4+ T cells. CD4+ T cells were stained with Fluo-4 and cytosolic Ca^{2+} was measured over a time course of 5 min in either unstimulated or stimulated (with anti-CD3/CD28 crosslinked antibodies) T cells. Unstimulated cells were treated with HBSS, $P2Y_{11}$ inhibitor or agonist (both 20 µM), H89 (10 µM), cAMP-AM (10 µM) or nocodazole (5 µM). All treatments were added after 45 s of imaging (frame 15). (A) Changes in Ca^{2+} signal (fold-change) over time plotted for each inhibitor as mean ± SE of 3 individual experiments with approx. 10-20 cells measured per experiment and treatment. (B) Changes in basal Ca^{2+} signaling, 5 min after the addition of the mentioned modulators, depicted as fold-change of start. (C) Ca^{2+} signal at the time point of the control peak (Minute 8:00 – 8:15) depicted as fold-change of control (*$p < 0.05$ vs. control, one-way ANOVA).

3.6 $P2Y_{11}$ signaling is necessary for T cell proliferation

Activated T cells proliferate to increase the number of cells responding to the respective antigen. This is a pivotal process for an effective immune response [30]. In contrast to Ca^{2+} signaling, it is a process very far downstream from the TCR-MHC contact. The effects of $P2Y_{11}$ on proliferation were investigated to prove that the receptor not only induces minimal changes in signaling cascades, but also influences the final outcome of T cell activation.

Figure 13A shows the gating strategy for the proliferation experiments. CD4+ cells were identified and then examined for CFSE fluorescence intensity. In this setting, lower fluorescence values mean higher proliferation since the stain is diluted upon cell division. The fluorescence histograms of selected treatments can be seen in **Figure 13B.** It shows that the $P2Y_{11}$ inhibitor visibly reduces T cell proliferation to 79% of the control. H89 has an even stronger effect (58%) and nocodazole reduces proliferation to 43%. This can also be observed in **Figure 13C**, which shows the statistical analysis of all proliferation experiments. While the $P2Y_{11}$ agonist decreases proliferation even further than the inhibitor, cAMP does not cause significant changes. Similarly, the A_{2A} inhibitor has no significant effect, although the agonist slightly reduces proliferation (80%). Since both the $P2Y_{11}$ inhibitor and agonist reduce proliferation – as a parameter for adequate T cell activation – it is possible that the receptor can have differential effects on T cells depending on its level of activation.

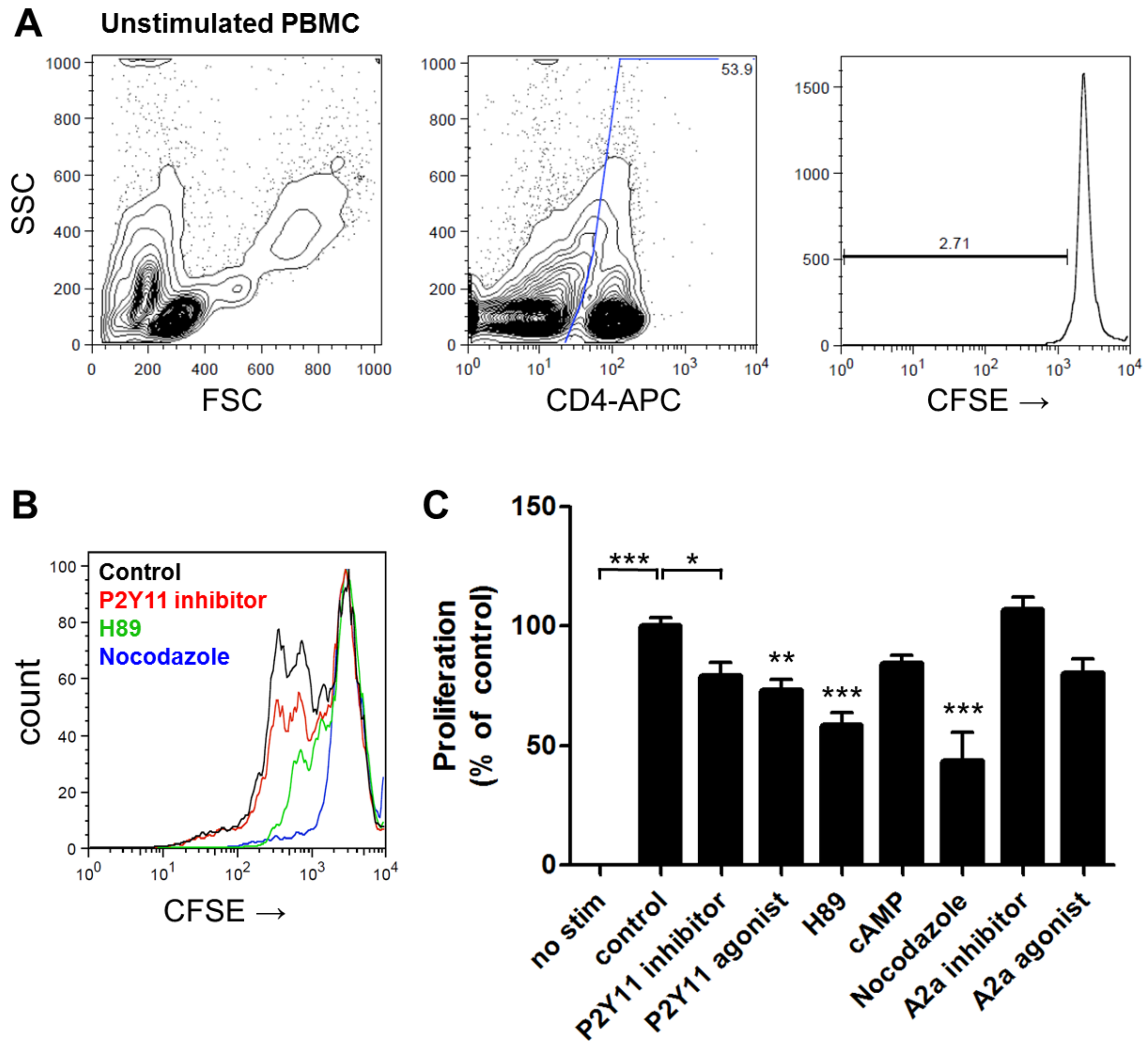


Figure 13: $P2Y_{11}$ modulates CD4+ T cell proliferation. (A) Gating strategy for proliferation experiments. The left panel shows the forward/side scatterplot. The middle panel depicts the gating of CD4+ cells and the right panel depicts the gating for CFSE dilution. (B) Overlay of histograms of the control, the $P2Y_{11}$ inhibitor (20 µM), H89 (10 µM) and nocodazole (5 µM). (C) Statistical analysis of all treatments: $P2Y_{11}$ inhibitor/agonist, H89, cAMP (10 µM), nocodazole and the A_{2A} inhibitor (1 µM) and agonist (100 nM). Data shown as mean ± SE (*$p < 0.05$ vs. control, 1-way ANOVA).

4 Discussion

T cell suppression in trauma and sepsis patients is a dangerous clinical complication and still not fully understood. Together with the initial hyperinflammatory response it leads to immunoparalysis and a greater susceptibility to infections [5], [7]. A promising approach to lift the curtain on the underlying mechanisms is to investigate the role of purinergic signaling in T cells. The effect of extracellular ATP on immune cells is highly dependent on their composition of purinergic receptors, as well as ectonucleotidases: ATP that directly activates P2 receptors can have completely opposite effects than when it is degraded and activates adenosine receptors [78]. In this context, ATP is generally regarded as pro-inflammatory, while adenosine triggers mainly anti-inflammatory pathways. It has repeatedly been shown that this ATP-based signaling network mediates various immune cell functions, ranging from the promotion of neutrophil chemotaxis to the regulation of macrophage activity [128], [138]. Within the past few years, purinergic signaling has also been discovered to mediate essential T cell functions. Evidence suggests that CD4+ T cells rely on basal purinergic signaling to maintain their metabolism in order to be ready for fast and efficient activation [173]. T cells use an intricate purinergic signaling loop in order to amplify the weak stimulatory signals generated from a TCR-MHC contact [142], [144]. This theory is supported by the fact that several purinergic components such as P2X1, P2X4 and Panx1 translocate to the immune synapse that forms between T cells and APCs [143], [144]. ATP release by Panx1 upon stimulation induces P2X receptor-mediated Ca^{2+} influx, which fuels mitochondrial production of ATP that is subsequently released [54], [173]. Furthermore, the P2X4 receptor has only recently been demonstrated to be a regulator of stimulated CD4+ T cell migration [145]. In this thesis it was hypothesized that P2X4 and $P2Y_{11}$ might operate in a coordinated manner, since they are both among the most highly expressed purinergic receptors in T cells [149]. $P2Y_{11}$ is a GPCR that, depending on the coupled G protein, works either via Ca^{2+} signaling ($G\alpha_{q/11}$) or cAMP production ($G\alpha_s$), the latter being the predominant downstream effector [169]. Similarly to P2X4, $P2Y_{11}$ inhibition could also reduce migration in both Jurkat and primary CD4+ T cells. The inhibition of its downstream effector PKA decreased migration as well. However, in contrast to P2X4, $P2Y_{11}$ does not accumulate at the leading edge but rather at the uropod of migrating T cells, which led to the hypothesis that it might mediate the translocation of P2X4 to the leading edge and therefore indirectly guide migration. It was shown that the colocalization of P2X4 and the mitochondria at the leading edge of Jurkat cells could be hampered upon inhibition or overstimulation of $P2Y_{11}$, as well as the inhibition of PKA. Mitochondrial activity was not altered by any of the $P2Y_{11}$-related modulators, suggesting that the receptor regulates the intracellular location of mitochondria, rather than their activity. These data suggest that $P2Y_{11}$ and P2X4 work in concert to guide the migration of stimulated T cells.

It was further theorized that $P2Y_{11}$ did not only affect certain pathways during migration but also in immune synapse formation of T cells with APCs. Unexpectedly, the experiments

show that none of the used $P2Y_{11}$ modulators could significantly alter the initial formation of immune synapses between T cells and anti-CD3-treated monocytes, or Jurkat cells with anti-CD3/CD28-coated beads. Only the inhibition of actin polymerization led to a substantial decrease in synaptic cells, validating that actin is indeed a necessary component of synapse formation and stability. Although the initial binding event between T cells and APCs is not influenced by $P2Y_{11}$, it was demonstrated that mitochondrial translocation to the immune synapse, an essential feature of T cell activation, is actually dependent on correct $P2Y_{11}$ signaling. Not only its inhibitor and agonist, but also cAMP and the PKA inhibitor significantly hampered the movement of mitochondria towards the immune synapse. The microtubule inhibitor nocodazole had the strongest effect by reducing mitochondrial motility in synaptic T cells to almost zero, suggesting that the $P2Y_{11}$ receptor might have direct effects on the mitochondrial motor machinery that connects the essential organelles to the microtubules [177]–[179]. While the underlying mechanisms of mitochondrial trafficking have been well-studied in neurons, very little is known about them in immune cells [16], [54]. As mentioned before, the mitochondria, amongst other functions, provide the ATP necessary for the purinergic signal amplification loop in T cells, further strengthening the argument that this process could be indirectly mediated by another purinergic component.

Previous studies have shown that antigen recognition and T cell function are influenced by mitochondrial ATP production as well as several purinergic components, such as P2X1, P2X4, P2X7 [54], [143], [144]. In order to test if $P2Y_{11}$ has an influence on essential T cell functions, one immediate and one long-term parameter were chosen to be measured. Regarding the immediate downstream effects, it was investigated how $P2Y_{11}$ would influence basal and stimulated cytosolic Ca^{2+} signaling. Interestingly, basal cytosolic Ca^{2+} levels were only significantly altered by the addition of cAMP, which is possibly induced via the cAMP-dependent PKA [180]. Control T cells showed a characteristic spike in cytosolic Ca^{2+} approximately 2 min after stimulation, in contrast to neutrophils which show a response within seconds [181]. This might be partially explained by the fact that purinergic receptors need to colocalize with the mitochondria prior to their stimulation and subsequent Ca^{2+} influx. Both the $P2Y_{11}$ and PKA inhibitors decreased the intensity of the Ca^{2+} spike upon stimulation, possibly by hampering the colocalization of mitochondria and P2X4, which led to a lower activation of P2X4 and to an accordingly lower Ca^{2+} influx. The agonist did not significantly inhibit Ca^{2+} signaling, although a trend is clearly visible. Treatment with cAMP led to a lowered peak upon stimulation even though it increased the basal signal. The cross-talk between Ca^{2+} and cAMP has been studied in several cell types, but is still not fully understood [180], [182]–[185]. However, the spatiotemporal control of both second messengers seems to be highly influential on their respective intertwined pathways, which simultaneously makes it more difficult to investigate their relation.

A rather long-termed parameter of T cell function is proliferation. Activation of T cells stimulates cell division and proliferation to ensure a large enough population of antigen-

specific T cells that can eliminate infected or cancerous target cells. This is, however, only established by optimal TCR signaling and downstream cascades. Thus, both inhibition and overstimulation of $P2Y_{11}$ could significantly reduce the T cells' ability to proliferate. The PKA inhibitor led to even stronger inhibition, which was to be expected since the previous experiments have shown that PKA is necessary for many cellular processes in this context. PKA is actually known for blocking TCR signaling, so one would expect its inhibition to work in a pro-inflammatory manner, but it is increasingly being recognized that the intracellular location of the cAMP-dependent PKA might have a big influence on differential outcomes [186]. The addition of cAMP itself had no significant effect on proliferation, possibly due to the fact that it is quickly metabolized and can therefore only transiently affect activation. The microtubule inhibitor expectedly reduced proliferation tremendously, since the trafficking of many organelles and signaling molecules is dependent on microtubules and vesicular transport [187].

Notably, the $P2Y_{11}$ agonist had very similar effects as the inhibitor in all experiments except Ca^{2+} signaling. In most cases, its effect was even stronger than the inhibitor's. This suggests that not only inhibition but also overstimulation of this receptor has detrimental effects on T cell activation. Such an overstimulation could also happen through excessive ATP release from damaged tissue, for example in sepsis [16]. Physiological extracellular ATP levels are typically in the nanomolar range, but an increase of up to 10 µM has previously been shown in septic mice [188]. This could be one of many mechanisms playing a role in trauma- and sepsis-related T cell suppression.

It also has to be remarked that the A_{2A} modulators had very similar effects as the $P2Y_{11}$ in mitochondrial translocation and T cell proliferation. As already mentioned, adenosine is generally considered as a negative regulator of the immune response and inhibitor of extensive inflammatory tissue damage, with the A_{2A} receptor being its primary anti-inflammatory effector [189], [190]. However, as mentioned before, the exact intracellular localization of signaling molecules can determine the outcome of a complex process like T cell activation. There are similarities to the $P2Y_{11}$ inhibitor, since A_{2A} is also coupled to $G\alpha_s$ and operates via cAMP. Considering the fact that the $P2Y_{11}$ receptor could not be identified in mice and other rodents, it seems likely that another purinergic receptor carries out its respective functions [169]. The A_{2A} receptor could be a possible candidate.

In conclusion, it was shown that the $P2Y_{11}$ receptor indirectly mediates CD4+ T cell migration, likely by supporting the colocalization of mitochondria with the P2X4 receptor. Similarly, also the translocation of mitochondria to the immune synapse is dependent on functional $P2Y_{11}$ signaling. This suggests that the downstream effectors of $P2Y_{11}$ work on the mitochondrial motor machinery. While the receptor does not influence the formation of immune synapses between T cells and APCs, it is necessary for correct downstream signaling of the TCR, since not only the immediate Ca^{2+} response but also long-term

functions like proliferation were altered by inhibition or overstimulation of $P2Y_{11}$. Although some functions have been elucidated, the exact downstream mechanisms remain yet to be understood. Further research could focus on how the $P2Y_{11}$ receptor affects the mitochondrial motor machinery, including proteins like Miro1, Trak and PINK1. Simultaneously, the connection to the A_{2A} receptor needs to be further evaluated. If it indeed has similar functions in mice as the $P2Y_{11}$ receptor has in humans, this would greatly facilitate the design of preclinical studies involving possible therapeutic targets.

This work shows that immoderate activation or inhibition of the $P2Y_{11}$ receptor has a great impact on mechanisms associated with T cell function. This pathway might be one of many that are dysregulated during excessive sepsis-related tissue damage and high ATP release. Further research on the underlying and related pathways could change our perspective on sepsis and lead to the development of effective treatments. Similarly, the modulation of these pathways might be used in an adverse manner to regulate the unwanted immune response to donor organs.

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List of Abbreviations

3D	Three-Dimensional
AIDS	Acquired Immunodeficiency Syndrome
ALS	Amyotrophic Lateral Sclerosis
AP	Alkaline Phosphatase
AP1	Activating Protein 1
APC	Antigen Presenting Cell
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
cAMP	Cyclic Adenosine Monophosphate
CBL	Casitas B Lineage Lymphoma
CFSE	Carboxyfluorescein Succinimidyl Ester
CNT	Concentrative Nucleotide Transporter
cSMAC	Central Supramolecular Activation Cluster
cTECs	Cortical Thymic Epithelial Cells
DAMP	Damage-Associated Molecular Pattern
DN	Double Negative
DP	Double Positive
dSMAC	Dorsal Supramolecular Activation Cluster
EGFP	Enhanced Green Fluorescent Protein
ENPP	Ectonucleotide Pyrophosphatase/Phosphodiesterase
ENT	Equilibrative Nucleoside Transporter
ENTPD	Ectonucleoside Triphosphate Diphosphohydrolase
FATPase	F0F1 ATP synthase
FITC	Fluorescein Isothiocyanate
FCS	Fetal Calf Serum
fMLP	N-Formylmethionine-leucyl-phenylalanine
FPR1	Formyl Peptide Receptor 1
GPCR	G Protein Coupled Receptor
GPIIb/IIIa	Glycoprotein IIb/IIIa
HBSS	Hank’s Balanced Salt Solution
HIV	Human Immunodeficiency Virus
HSC	Hematopoietic Stem Cell
ICAM-1	Intercellular Adhesion Molecule 1
IFN-γ	Interferon-γ
IL-2	Interleukin 2
IP3	Inositol 1,4,5-trisphosphate

IS	Immune Synapse
ITAM	Immune receptor Tyrosine-based Activation Motif
K^+	Potassium
LFA-1	Lymphocyte-Function Associated Antigen 1
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
Miro	Mitochondrial Rho-GTPase
MODS	Multiple Organ Dysfunction Syndrome
mTEC	Medullary Thymic Epithelial Cells
MTOC	Microtubule Organizing Center
NA	Numerical Aperture
NFAT	Nuclear Factor of Activated T Cells
NF-κB	Nuclear Factor κ B
NK	Natural Killer (Cells)
OMM	Outer Mitochondrial Membrane
Panx	Pannexin
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PINK	PTEN-Induced Putative Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCγ1	Phospholipase Cγ1
pSMAC	Peripheral Supramolecular Activation Cluster
PTEN	Phosphate and Tensin Homologue
PTK	Protein Tyrosine Kinase
ROS	Reactive Oxygen Species
SDF	Stromal Derived Factor
SE	Standard Error
siRNA	Small Interfering RNA
SMAC	Supramolecular Activation Cluster
TCR	T Cell Receptor
TNF-β	Tumor Necrosis Factor β
Trak	Trafficking Protein (kinesin-binding)
TRITC	Tetramethylrhodamine isothiocyanate
UDP	Uracil Diphosphate
UTP	Uracil Triphosphate
WHO	World Health Organization